



AD

RCS MEDDH - 288 ( RI )

# RESEARCH IN BIOLOGICAL AND MEDICAL SCIENCES

Including

BIOCHEMISTRY, COMMUNICABLE DISEASE AND IMMUNOLOGY, INTERNAL MEDICINE, PHYSIOLOGY, PSYCHIATRY, SURGERY, AND VETERINARY MEDICINE

Volume I.

ANNUAL PROGRESS REPORT 1 July 1976 - 30 September 1977

VOLUME

WALTER REED ARMY INSTITUTE OF RESEARCH WALTER REED ARMY MEDICAL CENTER WASHINGTON, D.C. 20012

AD AO 63402

pproved for public release; distribution unlimited. lestroy this report when no longer needed. Do not return it to the originator.

368 450

01 10 068

[PII Redacted]

3

UNCLASSIFIED
SECURITY CLASSIFICATION OF THIS PAGE (When Date Entered)

REPORT DOCUMENTATION		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVY ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
Research in Biolog Sciences including Biochemistry, Disease and Immunology, Internal	Communicable	5. TYPE OF REPORT & PERIOD COVERE Annual Progress Report-Vol 1 July 1976-30 September 19
Physiology, Psychiatry, Surgery a Veterinary Medicine 7. Author(s)	and	6. PERFORMING ORG. REPORT NUMBER N/A 8. CONTRACT OR GRANT NUMBER(e)
Listed at beginning of each work	unit report	N/A
PERFORMING ORGANIZATION NAME AND ADDRES	<u> </u>	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS
Walter Reed Army Institute of Res Walter Reed Army Medical Center	search	Listed at beginning of each report
Washington, D.C. 20012 1. CONTROLLING OFFICE NAME AND ADDRESS		12. REPORT DATE
U.S. Army Medical Research & Deve	elopment Command	September 1977
Washington, D.C. 20314		13. NUMBER OF PAGES
4. MONITORING AGENCY NAME & ADDRESS(If differ	ent from Controlling Office)	15. SECURITY CLASS. (of this report)
N/A		UNCLASSIFIED
		15. DECLASSIFICATION/DOWNGRADING SCHEDULE N/A
7. DISTRIBUTION STATEMENT (of the abetract entere $N/A$	ed in Block 20, il different fro	m Report)
8. SUPPLEMENTARY NOTES N/A		
9. KEY WORDS (Continue on reverse side if necessary	and identify by block number)	
Medical Sciences Into Biochemistry Phys	unology ernal Medicine siology chiatry	Surgery Veterinary Medicine
The various subjects covered in Contents. Abstracts of the indi	this report are li vidual investigati	isted in the Table of

DD 1 JAN 73 1473 EDITION OF T NOV 65 IS OBSOLETE

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE (When Date Entered)

79 01 10 063

RCS MEDDH-288(R1)

### RESEARCH IN BIOLOGICAL AND MEDICAL SCIENCES

#### INCLUDING

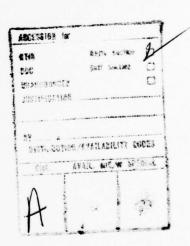
BIOCHEMISTRY, COMMUNICABLE DISEASE AND IMMUNOLOGY, INTERNAL MEDICINE, PHYSIOLOGY, PSYCHIATRY, SURGERY, AND VETERINARY MEDICINE

(Projects and work units are listed in Table of Contents)

ANNUAL PROGRESS REPORT
1 July 1976 - 30 September 1977

VOLUME I

WALTER REED ARMY INSTITUTE OF RESEARCH WALTER REED ARMY MEDICAL CENTER Washington, D.C. 20012



Approved for public release; distribution unlimited.

Destroy this report when no longer needed. Do not return it to the originator.

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

The FY 7T report is included in this Annual Progress Report

#### SUMMARY

The various subjects covered in this report are listed in the Table of Contents. Abstracts of the individual investigations are included on the DD Form 1498 introducing each work unit report, and names of investigators are given at the beginning of each report.

#### FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences - National Research Council.

# TABLE OF CONTENTS

# VOLUME I

			PAGE
3A161101A91C	IN-H	OUSE LABORATORY INDEPENDENT RESEARCH	
	095	Investigations of the owl monkey as a laboratory animal	1
	120	Antigenic components of the cell wall of	22
	194	Neisseria meningitidis Development of an organ culture method	22
	196	from intestinal biopsies Biochemical characterization of arbovirus	52
		antigens	59
	197	Molecular biology of rickettsia	66
	200		94
	201	Fibrinalysis in peripheral blood vessels	101
	202	Antigenic composition of Trypanosomes	106
	206	Factors influencing secretory states	136
	207	Model systems for antiparasitic drugs	144
3M161102BS01			
3M161102BS01	RESE	ARCH ON MILITARY DISEASES	
	121	Ecology and control of disease vectors	
		and reservoirs	166
	122	Basic pharmacological studies	212
	123	Biochemical research on cellular injury	235
	124	Biochemical research on military diseases	246
	125	Military stress: Circadian and ultradian factors	272
	126	Military psychiatry epidemiology	292
	127	Biological modulation of military	
		performance	299
	128	Mechanism of response to military stress	313
	129		324
	130		405
	131	Characteristics of attenuated dengue viruses	472
	132		495
	133		526
	134	Immunological mechanisms in microbial	
		infections	556
	135	Mechanisms of transmission of hepatitis viruses	564
	136	Development of biological products	571

137				PAGE
138		137	Pathologic manifestations of zoonotic	
139   Micobial genetics and taxonomy   629     140   Military hematology   640     141   Pathogenesis of renal diseases of military importance   664     142   Pathophesiology of systemic responses to shock and trauma   679     143   Gastrointestinal responses to shock and trauma   686     144   Control mechanisms of regional circulation   689			diseases of military importance	596
140   Military hematology   141   Pathogenesis of renal diseases of military importance   664   142   Pathoghesiology of systemic responses to shock and trauma   679   143   Gastrointestinal responses to shock and trauma   686   144   Control mechanisms of regional circulation   689				
141				629
importance   664   142   Pathophesiology of systemic responses to shock and trauma   679   143   Gastrointestinal responses to shock and trauma   686   144   Control mechanisms of regional circulation   689      VOLUME II				640
Shock and trauma		141		664
143 Gastrointestinal responses to shock and trauma 686 144 Control mechanisms of regional circulation 689    VOLUME II		142		679
VOLUME II     VOLUME II   VOLUME AND TROPICAL DISEASES   001   Epidemiologic studies of military diseases   729   003   Histopathologic manifestations of military diseases and injuries   741   VOLUME   VOLUM		143	Gastrointestinal responses to shock and	
MILITARY PREVENTIVE MEDICINE AND TROPICAL DISEASES  001 Epidemiologic studies of military diseases 699 002 Pathogenesis of Enteric Diseases 729 003 Histopathologic manifestations of military diseases and injuries 741 004 Epidemiology of hepatitis in the military 770 005 Gastrointestinal diseases of military importance 773 006 Rickettsial diseases of military personnel 792 007 Field studies of rickettsioses and other tropical diseases 839 008 Tropical and subtropical diseases in military medicine 948 009 Anti-schistosomal drug development 1071 010 Disease transmission in tropical populations 1077 011 Health care and management of laboratory animals 1189 012 Diseases of the military dog 1238  3M762770A803 MALARIA PROPHYLAXIS AND TREATMENT 081 Host responses to malaria 1252 082 Biological studies of insect infection and disease transmission 1265 083 Protective immunity in protozoan diseases 1284 084 Syntheses of anti-malarial drugs 1292 086 Biological evaluation of anti-malarial		144		
MILITARY PREVENTIVE MEDICINE AND TROPICAL DISEASES  001 Epidemiologic studies of military diseases 699 002 Pathogenesis of Enteric Diseases 729 003 Histopathologic manifestations of military diseases and injuries 741 004 Epidemiology of hepatitis in the military 770 005 Gastrointestinal diseases of military importance 773 006 Rickettsial diseases of military personnel 792 007 Field studies of rickettsioses and other tropical diseases 839 008 Tropical and subtropical diseases in military medicine 948 009 Anti-schistosomal drug development 1071 010 Disease transmission in tropical populations 1077 011 Health care and management of laboratory animals 1189 012 Diseases of the military dog 1238  3M762770A803 MALARIA PROPHYLAXIS AND TREATMENT 081 Host responses to malaria 1252 082 Biological studies of insect infection and disease transmission 1265 083 Protective immunity in protozoan diseases 1284 084 Syntheses of anti-malarial drugs 1292 086 Biological evaluation of anti-malarial				
001 Epidemiologic studies of military diseases 729 002 Pathogenesis of Enteric Diseases 729 003 Histopathologic manifestations of military diseases and injuries 741 004 Epidemiology of hepatitis in the military 770 005 Gastrointestinal diseases of military importance 773 006 Rickettsial diseases of military personnel 792 007 Field studies of rickettsioses and other tropical diseases 839 008 Tropical and subtropical diseases in military medicine 948 009 Anti-schistosomal drug development 1071 010 Disease transmission in tropical populations 1077 011 Health care and management of laboratory animals 1189 012 Diseases of the military dog 1238  3M762770A803 MALARIA PROPHYLAXIS AND TREATMENT 081 Host responses to malaria 1252 082 Biological studies of insect infection and disease transmission 1265 083 Protective immunity in protozoan diseases 1284 084 Syntheses of anti-malarial drugs 1292 086 Biological evaluation of anti-malarial			VOLUME II	
002 Pathogenesis of Enteric Diseases 003 Histopathologic manifestations of military diseases and injuries 741 004 Epidemiology of hepatitis in the military 005 Gastrointestinal diseases of military importance 773 006 Rickettsial diseases of military personnel 007 Field studies of rickettsioses and other tropical diseases 839 008 Tropical and subtropical diseases in military medicine 948 009 Anti-schistosomal drug development 1071 010 Disease transmission in tropical populations 1077 011 Health care and management of laboratory animals 1189 012 Diseases of the military dog 1238  3M762770A803 MALARIA PROPHYLAXIS AND TREATMENT 081 Host responses to malaria and disease transmission 265 083 Protective immunity in protozoan diseases 084 Syntheses of anti-malarial drugs 1292 086 Biological evaluation of anti-malarial	3M762770A802	MILI	TARY PREVENTIVE MEDICINE AND TROPICAL DISEAS:	ES
002 Pathogenesis of Enteric Diseases 003 Histopathologic manifestations of military diseases and injuries 741 004 Epidemiology of hepatitis in the military 005 Gastrointestinal diseases of military importance 773 006 Rickettsial diseases of military personnel 007 Field studies of rickettsioses and other tropical diseases 839 008 Tropical and subtropical diseases in military medicine 948 009 Anti-schistosomal drug development 1071 010 Disease transmission in tropical populations 1077 011 Health care and management of laboratory animals 1189 012 Diseases of the military dog 1238  3M762770A803 MALARIA PROPHYLAXIS AND TREATMENT 081 Host responses to malaria and disease transmission 265 083 Protective immunity in protozoan diseases 084 Syntheses of anti-malarial drugs 1292 086 Biological evaluation of anti-malarial		001	Epidemiologic studies of military diseases	699
003		002		
004 Epidemiology of hepatitis in the military 005 Gastrointestinal diseases of military importance 773 006 Rickettsial diseases of military personnel 792 007 Field studies of rickettsioses and other tropical diseases 839 008 Tropical and subtropical diseases in military medicine 948 009 Anti-schistosomal drug development 1071 010 Disease transmission in tropical populations 1077 011 Health care and management of laboratory animals 1189 012 Diseases of the military dog 1238 189 1238 1806 1906 Biological studies of insect infection and disease transmission 1265 083 Protective immunity in protozoan diseases 1284 084 Syntheses of anti-malarial drugs 1292 086 Biological evaluation of anti-malarial		003	Histopathologic manifestations of military	741
importance 773  O06 Rickettsial diseases of military personnel 792  O07 Field studies of rickettsioses and other tropical diseases 839  O08 Tropical and subtropical diseases in military medicine 948  O09 Anti-schistosomal drug development 1071  O10 Disease transmission in tropical populations 1077  O11 Health care and management of laboratory animals 1189  O12 Diseases of the military dog 1238  3M762770A803 MALARIA PROPHYLAXIS AND TREATMENT  O81 Host responses to malaria 1252  O82 Biological studies of insect infection and disease transmission 1265  O83 Protective immunity in protozoan diseases 1284  O84 Syntheses of anti-malarial drugs 1292  O86 Biological evaluation of anti-malarial		004		
006 Rickettsial diseases of military personnel 792 007 Field studies of rickettsioses and other tropical diseases 839 008 Tropical and subtropical diseases in military medicine 948 009 Anti-schistosomal drug development 1071 010 Disease transmission in tropical populations 1077 011 Health care and management of laboratory animals 1189 012 Diseases of the military dog 1238  3M762770A803 MALARIA PROPHYLAXIS AND TREATMENT 081 Host responses to malaria 1252 082 Biological studies of insect infection and disease transmission 1265 083 Protective immunity in protozoan diseases 1284 084 Syntheses of anti-malarial drugs 1292 086 Biological evaluation of anti-malarial			Gastrointestinal diseases of military	
007 Field studies of rickettsioses and other tropical diseases 839  008 Tropical and subtropical diseases in military medicine 948  009 Anti-schistosomal drug development 1071  010 Disease transmission in tropical populations 1077  011 Health care and management of laboratory animals 1189  012 Diseases of the military dog 1238  3M762770A803 MALARIA PROPHYLAXIS AND TREATMENT  081 Host responses to malaria 1252  082 Biological studies of insect infection and disease transmission 1265  083 Protective immunity in protozoan diseases 1284  084 Syntheses of anti-malarial drugs 1292  086 Biological evaluation of anti-malarial				
tropical diseases 839  008 Tropical and subtropical diseases in military medicine 948  009 Anti-schistosomal drug development 1071  010 Disease transmission in tropical populations 1077  011 Health care and management of laboratory animals 1189  012 Diseases of the military dog 1238  3M762770A803 MALARIA PROPHYLAXIS AND TREATMENT  081 Host responses to malaria 1252  082 Biological studies of insect infection and disease transmission 1265  083 Protective immunity in protozoan diseases 1284  084 Syntheses of anti-malarial drugs 1292  086 Biological evaluation of anti-malarial				792
008 Tropical and subtropical diseases in military medicine 948 009 Anti-schistosomal drug development 1071 010 Disease transmission in tropical populations 1077 011 Health care and management of laboratory animals 1189 012 Diseases of the military dog 1238  3M762770A803 MALARIA PROPHYLAXIS AND TREATMENT  081 Host responses to malaria 1252 082 Biological studies of insect infection and disease transmission 1265 083 Protective immunity in protozoan diseases 1284 084 Syntheses of anti-malarial drugs 1292 086 Biological evaluation of anti-malarial	**	007		839
009 Anti-schistosomal drug development 1071 010 Disease transmission in tropical populations 1077 011 Health care and management of laboratory animals 1189 012 Diseases of the military dog 1238  3M762770A803 MALARIA PROPHYLAXIS AND TREATMENT  081 Host responses to malaria 1252 082 Biological studies of insect infection and disease transmission 1265 083 Protective immunity in protozoan diseases 1284 084 Syntheses of anti-malarial drugs 1292 086 Biological evaluation of anti-malarial		800		040
010 Disease transmission in tropical populations 1077 011 Health care and management of laboratory animals 1189 012 Diseases of the military dog 1238  3M762770A803 MALARIA PROPHYLAXIS AND TREATMENT  081 Host responses to malaria 1252 082 Biological studies of insect infection and disease transmission 1265 083 Protective immunity in protozoan diseases 1284 084 Syntheses of anti-malarial drugs 1292 086 Biological evaluation of anti-malarial		009		
populations 1077  011 Health care and management of laboratory animals 1189  012 Diseases of the military dog 1238  3M762770A803 MALARIA PROPHYLAXIS AND TREATMENT  081 Host responses to malaria 1252  082 Biological studies of insect infection and disease transmission 1265  083 Protective immunity in protozoan diseases 1284  084 Syntheses of anti-malarial drugs 1292  086 Biological evaluation of anti-malarial				10/1
011 Health care and management of laboratory animals 1189 012 Diseases of the military dog 1238  3M762770A803 MALARIA PROPHYLAXIS AND TREATMENT  081 Host responses to malaria 1252 082 Biological studies of insect infection and disease transmission 1265 083 Protective immunity in protozoan diseases 1284 084 Syntheses of anti-malarial drugs 1292 086 Biological evaluation of anti-malarial				1077
012 Diseases of the military dog 1238  3M762770A803 MALARIA PROPHYLAXIS AND TREATMENT  081 Host responses to malaria 1252 082 Biological studies of insect infection and disease transmission 1265 083 Protective immunity in protozoan diseases 1284 084 Syntheses of anti-malarial drugs 1292 086 Biological evaluation of anti-malarial		011	Health care and management of laboratory	
3M762770A803 MALARIA PROPHYLAXIS AND TREATMENT  081 Host responses to malaria 1252 082 Biological studies of insect infection and disease transmission 1265 083 Protective immunity in protozoan diseases 1284 084 Syntheses of anti-malarial drugs 1292 086 Biological evaluation of anti-malarial		012		
081 Host responses to malaria 1252 082 Biological studies of insect infection and disease transmission 1265 083 Protective immunity in protozoan diseases 1284 084 Syntheses of anti-malarial drugs 1292 086 Biological evaluation of anti-malarial		012	Diseases of the military dog	1238
082 Biological studies of insect infection and disease transmission 1265 083 Protective immunity in protozoan diseases 1284 084 Syntheses of anti-malarial drugs 1292 086 Biological evaluation of anti-malarial	3M762770A803	MALA	RIA PROPHYLAXIS AND TREATMENT	
082 Biological studies of insect infection and disease transmission 1265 083 Protective immunity in protozoan diseases 1284 084 Syntheses of anti-malarial drugs 1292 086 Biological evaluation of anti-malarial		081	Host responses to malaria	1252
083 Protective immunity in protozoan diseases 1284 084 Syntheses of anti-malarial drugs 1292 086 Biological evaluation of anti-malarial			Biological studies of insect infection	
084 Syntheses of anti-malarial drugs 1292 086 Biological evaluation of anti-malarial		083		
086 Biological evaluation of anti-malarial				
				1292
				1297

			DAGE
	087	Determination of pharmacological effects	PAGE
		of anti-malarial drugs	1307
	880	Biochemical research on anti-malarials	1363
	089	Field studies on drug resistant malaria	1373
3E762771A804	MILI	TARY PSYCHIATRY	
	041	Behavioral variables in autonomic function	
		and disease in military personnel	1446
	042	Military preventive psychiatry	1452
	043	Military stress: Health, performance	
		and injury factors	1459
	044	Neuroendocrine response to military stress	1484
	045	Follow-up studies of human volunteers	
		who received psychoactive substances	1491
207627777005	WEGD		
3E762771A805	MICR	OWAVE INJURY PREVENTION AND TREATMENT	
	041	Biological interactions with and hazards	
		of microwave radiation	1494

Project 3A161101A91C

IN-HOUSE LABORATORY INDEPENDENT RESEARCH

0

RESEARCH AND TECHNOLOG	Y WORK UNIT S	UMMARY	TOWNS OF THE	CY ACCESSION®	77 10			CONTROL SYMBOL R&E(AR)636
76 10 01 D. Change	S. SUMMARY SCTY	6. WORK SECURITY	7. REGR	_	NL	ON TRACTO	R ACCESS	9. LEVEL OF SUM
10. NO./CODES:* PROGRAM ELEMENT		NUMBER		REA NUMBER	T		IT NUMBER	
- PRIMARY   61101A	3A161101A			00		0:	95	
b. CONTRIBUTING	1							
c. CONTRIBUTING								
11. TITLE (Precede with Security Classification Cod	•)*							
(U) Investigations of the	ne Owl Monk	ey as a Lab	orat	ory Anim	al			
12. SCIENTIFIC AND TECHNOLOGICAL AREAS								
001700 Animal Husbandry	O2600 B	TOLOGY U	1290	D Physio	logy	16. PERFOR	MANCE MET	HOD
				1	1		_	
76 01	CONT		DA	OURCES ESTIMA	75 4 9905	ESSIONAL MAN Y	In-Hou	DS (In thousands)
A DATES/EFFECTIVE: NA	EXPIRATION:		10. 123	PRECEDING	TE PAOP	ESSIONAL MAN 1		Do pri anoceanos)
b. NUMBER:*			FISCAL	77		1.5		134
C TYPE:	d AMOUNT:		YEAR	CURRENT				
e KIND OF AWARD:	f. CUM. AMT.			78		1.5		70
19. RESPONSIBLE DOD ORGANIZATION			20. PER	FORMING ORGAN	IZATION			
NAME: Walter Reed Army Institute of Research Division of Veterinary Resources								
				Division	of Vet	erinary I	Resour	ces
ADDRESS: Washington, DC 20012								
	PRINCIPAL INVESTIGATOR (Fumish SSAN II U.S. Academic Institution) NAME: Hall, Robert D., MAJ, VC							
RESPONSIBLE INDIVIDUAL Rapmund,	Garrison, C	OL, MC	NAME:				J, VC	
NAME:			TELEPHONE: 202 427-5430					
TELEPHONE: 202 576-3	3551		SOCIA	SECURITY ACC		R:		
21. GENERAL USE				TE INVESTIGAT	DRS			
Foreign Intelligence not	NAME:							
22, KEYWORDS (Precede EACH with Security Class)								
(U) Aotus trivirgatus;		nkey; (U)	Bree	ding; (	J) Kar	votype		
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25	PROGRESS (Furnish	individual paragraphs id	entitled by	number. Precede	text of each wi	th Security Classi	fication Code	1.)
23. (U) To obtain biol	ogic and ph	nysiologic (	data	of the o	wl monk	ey essen	tial f	for opti-
mizing colony management	procedures	, recogniz	inc d	isease s	tates a	ind breed	ing ar	nd raising
Aotus for studies of fal	ciparum ma	laria, tor i	which	1t 1s t	he best	nonhuma	n prin	nate host.
Aotus are no longer comm	ercially av	/ailable in	suff	icient r	umbers	to meet	the Ar	my's re-
quirements for its malar								
sently unavailable or un								
success of a domestic br 24. (U) Physiologic da								
clude the following meas	uromonte.	homatologic		rum bioc	homical	and on	VIDO C	s to III-
cycle, gestation period,								
be used.	and grower	i dila dever	opiner	c paccer		undur a p	loccut	ares will
25. (U) 76 10 - 77 09	In a 16 mg	onth period	the	WRAIR CO	lony ha	d 49 sin	ale li	ive births.
including the 2nd known								
ing pairs based on their								
breeding pairs, purporte								
Breeding pairs of marked	ly differer	nt ages and	weig	hts were	equall	y produc	tive.	The se-
quence and time of erupt	ion of deci	iduous and p	perma	nent tee	th were	establi	shed a	and a rate
of weight gain for infan	t monkeys w	vas ascerta	ined	and plot	ted. S	erum est	rogen	levels in-
dicated that the length	of the estr	rous cycle	is 16	days.	Commerc	ially av	ailabl	e Bolivian
owl monkeys were found t	o be suscep	otible to fa	alcip	arum mal	aria.	Serum el	ectrop	horesis
showed a high prevalence	of double	albumin, s	low m	oving al	bumin a	nd tripl	e alph	na
plobulin in owl monkeys.	Hematoloc	ic and seri	ım ch	emistry	values	word dif	ficul+	+0
interpret due to glomeru	Ionephritis	of unchara	acter	ized pre	valence	and sev	erity.	For
technical report see Wal 1 Jul 76 - 30 Sep 77.	ter Reed Ar	may. Institut	te of	Researc	h Annua	1 Progre	ss Rep	ort,
Available to contractors upon originator's app	roval.	1						
DD FORM 1498 PREVIOUS	EDITIONS OF T	HIS FORM ARL OF	OBSOLE	ETE.	MS 1498A. 1	NOV 65		

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 095 Investigations of the Owl Monkey as a Laboratory Animal

Investigators.

Principal: Robert D. Hall, MAJ, VC

Associates: David E. Davidson, LTC, VC, Michael J. Reardon, MAJ, VC, Robert J. Beattie, LTC, VC, George H. Wyckoff, Jr., LTC, VC

#### Description:

To obtain biologic and physiologic data on the owl monkey (Aotus trivirgatus) which are needed to optomize colony management, recognize disease states and successfully breed and raise these monkeys for falciparum malaria studies, for which it is the best nonhuman primate host.

During the reporting period, research activities have included analyses of: (1) specific parameters affecting reproduction, (2) a method for diagnosing pregnancy, (3) the length of the estrous cycle, (4) time and sequence of eruption of deciduous and permanent teeth and rate of weight gain of infant monkeys, (5) the susceptibility of Bolivian and monkeys to falciparum malaria, (6) serum protein electrophoretic patterns of owl monkeys, and (7) hematologic, serum chemical and urinalysis data.

#### Reproduction Data.

Ninty four (94) breeding pairs of owl monkeys were established during October and December of 1975 and August 1976. Pairs were established with mates of similiar body weight, age, and/or pelage coloration. Approximately 10% of these pairs (9 of 94) were temperamentally incompatable and were separated. Another 23 pairs were separated due to illness or death. Selected reproductive data are shown in Table 1 for 31 pairs of monkeys which have remained together for 22 to 24 months. Conceptions were detected by urine pregnancy tests (see below) and/or palpation. Twenty one single, viable offspring were produced by 17 of the 31 pairs, whereas 14 pairs did not produce offspring. Nine females never conceived (Row 1, column 2). Five females (Row 2, column 2) conceived a total of 16 times but the pregnancies terminated in 14 resorptions and 2 abortions. Only 2 resorptions were observed in monkeys which produced viable offspring.

Several parameters were examined with respect to the breeding performance of these monkeys. One of these was the karyotypes of the animals. Aotus have at least 4 different phenotypes, based on pelage, and 7 different karyotypes, with diploid (2n) chromosome numbers varying from 46 to 54 (1). Several authors have implied that it is important to match owl monkeys by karyotypes to produce live births (2,3). Monkeys in this study were paired before being karyotyped and remained paired as originally established to evaluate the productivity of various karyotypic matings. The karyotypes of 253 monkeys are shown in Table 2. Monkeys with karyotypes II, III, IV and V are phenotypically indistinguishable, originate from the same areas of South America, and make up over 80% of the monkeys in the WRAIR colony. Karyotypes II, III, and IV form a genetically related breeding group, with karyotype III being a hybrid of karyotypes II and IV (1). Karyotype III has 3 unpaired chromosomes which, theoretically, could result in greater difficulty in producing viable offspring. Little is known about the productivity of breeding pairs with markedly different types of chromosomes.

Table 3 shows the incidence of conceptions, births and abortions/resorptions for different karyotypic matings. Two pairs (Group A) had karyotypes with morphologically dissimiliar chromosomes; yet, both females conceived once each and the pregnancies lead to live births. Two of 3 other genetically "mismatched" pairs from the colony (not shown in this study group) have also produced offspring. Karyotypes of the infants are presently being studied. One of the hybrids is shown in Table 2 as having a diploid chromosome number of 49, different from either of its parents. Hybrids other than karyotype III have not been found among feral owl monkeys, suggesting that crossbreeding of subspecies in nature is rare. This may be due primarily to geographic separation. Data thus far does not support a premise that owl monkeys must be matched by compatable karyotype for first generation breeding success. However, hybrids have no current value as breeders until their productivity is demonstrated.

Both mates of the remaining 29 pairs shown on Table 3 (Groups B, C and D) had related karyotypes II, III or IV. Ten pairs (Group B) had genetically compatable karyotypes. Eight of these (90%) conceived a total of 15 times resulting in 8 live births and 7 abortions/resorption. Pairs with one hybrid (karotype III) parent (Group C) had 8 of 12 females (67%) conceive 16 times resulting in 6 live births and 10 resorptions. Breeding pairs with both karyotype III parents (Group D) had 4 of 7 (57%) females conceive 6 times, and had 5 live births and 1 resorption. The percentage of monkeys that conceived was highest (80%) in the group with compatible karyotypes (Group B), intermediate (67%) in the group with one karyotype

III parent (Group C), and lowest (57%) in the group with both karyotype III parents (Group D). Although the number of monkeys in each group is small, these data tend to support the premise that karyotype III monkeys with unpaired chromosomes have lower productivity. On the other hand, the percentage of conceptions that ended in resorption or abortion was 63% for the single hybrid parent group (Group C), 47% for compatible karyotype pairs (Group B), and only 17% for the group with both hybrid parents (Group D). Apparently the high abortion/resorption rate was not related to incompatable karyotypes.

Monkeys from the previously described 31 breeding pairs were segregated into age categories based on dental wear and evaluated for fertility and parity (Table 4). Monkeys were considered young adults if all permanent teeth were present, and all canines and molars had sharp points. Mature monkeys had worn canines and molars with rounded points. Fully mature monkeys had very worn teeth with flattened occlusal surfaces, and old monkeys had teeth which were worn to within 1 or 2 mm of the gingiva. Available information does not permit the translation of these age groups into calendar years. Twenty-two of the 31 males (71%) successfully impregnated their mates. The fertility rate was similiar for each age group except that only one old, nonfertile male was evaluated. Female fertility was lowest among the mature animals with only 3 of 8 (38%) mature females having conceived. This may have occurred by chance due to the small sample size. Age, therefore, did not appear to be an important factor affecting fertility. On the other hand, all 5 females which conceived but lost the fetus were young adults. Additional observations are necessary before any associations are made between abortions/resorptions and the age of the female. Productive and nonproductive monkeys had comparable body weights (Table 5).

Eleven breeding pairs of monkeys at WRAIR had their 2nd birth and 1 had its 3rd birth. The average interval between births was 16.5 months (range from 5 to 42 months). The minimum interbirth interval of 5 months strengthens our previous estimate, based on vaginal cytology, of 18 weeks as the length of gestation for this species. During the past year, the WRAIR had its first 2nd-generation owl monkey birth. The parents were both born 3 years and 1 month previously, were separated from their parents at 8 months of age, and were caged together since that time. This represents the second known 2nd-generation birth in captivity and demonstrates the ability of this species to produce offspring by 3 years of age.

A method for diagnosing pregnancy with minimal disturbance to the monkeys was needed. Urine samples were collected biweekly by placing a polyethylene bag under the cage floor of each breeding pair. Urine, voided by one or both animals of the pair, was tested for chorionic gonadotropin using the Sub-Human Primate Pregnancy Test kit (Ortho Diagnostics Inc., Raritan, NJ). The kit was originally designed for use with rhesus monkeys and has been used successfully with macaques, baboons, marmosets and apes. Whenever test results changed from negative to positive or equivocal, the testing frequency was increased to 2 or 3 times per week until results clearly showed consistently positive or negative reactions. The 31 pairs of monkeys previously described provided 1688 urine samples.

The results of the qualitative assays for chorionic gonadatropin are shown in Table 6. A correct negative or positive response was observed 88% of the time with 17 parous females, 5 fertile but nonparous females and 9 nonfertile females. False negative and false positive reactions were observed 2 to 5% of the time for these same groups. False negative results were probably caused by testing of, or dilution with, male urine. False positive results seem to have been related to the glomerulonephritis suspected of affecting many animals of the colony. Positive reactions were observed with urine from males with severe glomerulonephritis. Borderline reactions inherent to the test and urine biochemically modified by glomerulonephritis both may have contributed to the observed 6% equivocal responses from nonpregnant monkeys.

The sensitivity and specificity of the Sub-Human Primate Pregnancy Test kit are being evaluated by radioimmunoassay. Despite problems with occasional spurious positive or negative results, the urine pregnancy test is presently the best method for diagnosing pregnancy without periodically handling the monkeys. Most of the fetal resorptions observed by us would have been undetected without this technique. Early confirmation of pregnancy can be made by increasing the testing frequency to 3 times per week when an initial positive or equivocal result occurs. Consistently positive results for 2 or 3 consecutive weeks confirm pregnancy since false results are generally sporadic. If monkeys are tested biweekly, parturition can be estimated as  $100 \pm 10$  days from the first confirmed positive test result.

The length of the estrous cycle for owl monkeys was estimated to be  $14 \pm 4$  days based on observed changes in vaginal cytology (4). These data were inconclusive, however, since only 13 cycles were observed in 28 female monkeys over a 1 year study period. Attempts to extend or confirm these results were made by quantitating and plotting serum estrogen levels

for 5 adult, nonpregnant female monkeys over a 2 month period. Serum samples were obtained from each of the monkeys every 2 to 4 days and were assayed for estrone and estradiol by radioimmunoassay. One of the 5 monkeys had alternating high and low levels of estrone and estradiol at periodic intervals which suggested complete estrous cycles (Figure la). The intervals of time between 4 estradiol peaks was 17, 17 and 14 days. Two additional monkeys also had cycling estrogen levels, but the levels were not as marked as the first monkey and the time intervals between peaks were sporadic (Figure lb). Two monkeys on seroassay showed occasional high peaks of estrogen without evidence of periodicity. Clearly these 2 animals were not having regular ovulatory cycles. Although the reasons for absence of regular cycling is not known, one animal died of glomerulonephritis shortly after sampling was completed.

## 2. Development of infant owl monkeys.

Data on the time and sequence of eruption of deciduous and permanent teeth of owl monkeys were expanded by examining newborn monkeys twice weekly until all deciduous teeth had erupted and biweekly until all permanent teeth had erupted. The mean eruption times for deciduous and permanent teeth respectively are shown in Tables 7 and 8. These data provide a useful basis for estimating the age of infant or juvenile owl monkeys.

In our experience, monitoring body weight was the simplest method for evaluating the health and monitoring the development of infant Aotus. Body weights were obtained when dental examinations were performed and a weight chart (Figure 2) was plotted. The body weight of 36 apparently normal, 1-to-3 day old owl monkeys ranged from 69 to 114 grams with an average of  $90 \pm 11.2$  grams (+ 1 standard deviation). The rate of weight gain was approximately 16 grams per week from birth through the 15th week of age and 10 grams per week thereafter through the 52nd week. This weight gain chart provided a valuable standard with which to evaluate the health status and observed versus expected growth performance for all other infant Aotus. No such standard has been here-to-fore available for any breeding colony.

# 3. Malaria susceptibility.

In late 1976 owl monkeys became commercially available in limited numbers from Bolivia. Their susceptibility to falciparum malaria had not previously been tested. Karyotypically these animals are distinct (Karyotype VI). However, their pelage coloration closely resembles owl monkeys from

Brazil (Karyotype I). The latter are reported to be more resistant to malaria infection than the commonly used Colombian owl monkey (5). The WRAIR acquired 7 Bolivian owl monkeys and evaluated their susceptibility to malaria infection. The Viet Nam Smith strain, a poly-drug resistant, monkey adapted strain, of <u>Plasmodium falciparum</u> was serially passed through splenectomized and then intact Bolivian monkeys. Three splenectomized monkeys developed 18% to 34% parasitemias and died; 4 intact monkeys developed 3% to 18% parasitemias and 2 died. The severity and duration of malaria infection and its outcome in Bolivian owl monkeys were similar to that which occurs in Colombian monkeys.

## 4. Serum Protein Electrophoresis.

Bisalbuminemia and triple alpha globulins have been observed in owl monkeys by several authors (6,7,8). The number of animals examined, however, has been small and interpretations of the results are in conflict. Based on the serum immunoelectrophoretic patterns of 5 monkeys, Brumback and Willenborg (6) demonstrated that karyotype I owl monkeys (Ma's classification) had 2 distinct albumin fractions whereas Colombian owl monkeys did not. Collins et al, (7) reported that 2 of 6 Colombian monkeys and 0 of 5 karyotype I monkeys had bisalbuminemia. Wellde et al, (7) found bisalbuminemia in 33 of 110 owl monkeys but did not relate these to karyotype. Collins et al, (7) also reported that all 5 Brazilian (Karyotype I) monkeys had 3 alpha globulins whereas the 6 Colombian monkeys had only 2 alpha globulins. Serum protein electrophoresis was performed on specimens from owl monkeys with known karyotypes in order to investigate whether or not specific biochemical patterns were associated with one or more karyotypes.

Fifteen of 21 (71%) karyotype I monkeys had triple alpha globulins, (Table 9) whereas karyotype II, III and IV animals had a much lower prevalence (13% to 25%). Additionally, all karyotype I animals had a single expected albumin whereas 33% to 44\$ of the karyotype II, III and IV monkeys had 2 distinct albumin peaks. Twelve monkeys with karotypes II, III and IV had single albumins which corresponded to the albumin having the slower electrophoretic mobility and the remaining 69 monkeys had the single faster moving albumin. The only Karyotype V monkey examined had bisalbuminemia. Three karyotype VI monkeys had the single slow moving albumin and 2 had triple alpha globulins. These results support the findings of Collins et al, that karyotype I monkeys do not have the slower moving albumin, and that they have a higher prevalence of triple alpha globulins than karyotypes II, III or IV. Although bisalbuminemia and triple alpha globulins occur with different frequencies in different karyotypes, the karyotype of an individual monkey cannot be ascertained by serum electrophoresis.

Other animals will be examined to complete this study. The genetic basis for the occurence of bisalbuminemia and triple alpha globulins will be studied with offspring from this colony.

## 8. Hematology and Chemistry Values

An important part of this research program has been to obtain hematologic and serum chemistry values for "normal" owl monkeys. This effort has been thwarted with the recognition that many of the monkeys in the WRAIR colony have glomerulonephritis. Over the past year glomerulonephritis was the primary cause of 15 of 25 adult monkey deaths, and lesions of glomerulonephritis were commonly found in animals which died of septicemia or other causes. A necropsy survey performed at the New England Regional Primate Research Center showed that 45 of 57 (79%) of the owl monkeys examined had various stages of glomerulonephritis (9). WRAIR monkeys which have died of renal disease have had severe to moderate anemia, elevated blood urea nitrogen and creatinine levels and/or depressed total serum protein and albumin levels. Numerous clinically normal appearing monkeys have also had altered hematologic and serum chemical values. Methods must be devised which will identify animals that are free of glomerulonephritis before normal baseline data can be obtained.

Glomerulonephritis often causes the excretion of large amounts of protein in the urine. Therefore, urinalysis may provide the easiest method for detecting glomerulonephritis. A total of 640 urinalyses were performed with N-Multistix (Ames Co., Division of Miles Laboratories Inc., Indiana) on specimens from 25 apparently healthy, adult female monkeys. The concentration of urinary protein for each of the 25 monkeys consistently showed: (1) 4 with  $\leq$  30 mgm %; (2) 12 with 30 to 100 mgm %; and (3) 9 with 100 to 300 mgm %. Whether or not the severity of glomerulonephritis is directly related to protein concentration in the urine remains to be proven. If shown, however, urine protein analysis may be the easiest method for detecting and monitoring this disease.

#### Summary.

The necessity to pair owl monkeys by karyotype to achieve breeding success was challenged. Paired monkeys with morphologically dissimilar chromosomes produced hybrid offspring. Resorptions and abortions were not related to karyotype. Instead, all 5 nonparous females that had resorptions or abortions were young adults. The second known captive-born 2nd-generation birth occurred at the WRAIR. Clearly this species

can produce offspring by 3 years of age. The Sub-Human Primate Pregnancy Test kit, originally designed for use with rhesus monkeys, was useful in diagnosing pregnancy. Serum estrone and estradiol levels in 1 of 5 monkeys substantiated earlier findings, based on vaginal cytology, that the length of the estrous cycle for owl monkeys was approximately 16 days. Data on the time and sequence of eruption of deciduous and permanent teeth were expanded, and a weight gain chart was constructed as a standard by which to evaluate the health and expected growth of infants. Bolivian owl monkeys were found to be susceptible to falciparum malaria. Serum protein electrophoresis showed a higher prevalence of triple alpha globulins in Brazilian owl monkeys (71%) than in Colombian monkeys (13% to 25%) and a 30-40% prevalence of bisalbuminemia in Colombian owl monkeys compared with a 0% incidence in Brazilian monkeys. The interpretations of normal hematology and serum chemistry values was compromised with the recognition of glomerulonephritis as a common disease in the colony.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 095 Investigations of the Owl Monkey as a Laboratory Animal

## Literature Cited:

#### References:

- Ma, N.S.F., Jones, T.C., Miller, A.C., et. al. 1976. Chromosome Polymorphism and Banding Patterns in Owl Monkeys (Aotus). Lab Anim Sci 26(6):1022-1036.
- Elliott, M.W., Sehgal, P.K., and Chalifoux, L.V. 1976. Management and Breeding of <u>Aotus trivirgatus</u>. Lab Anim Sci 26(6) 1037-1040.
- Cicmanec, J.C. and Campbell, A.K. 1977. Breeding the Owl Monkey (Actus trivirgatus) in a Laboratory Environment. Lab Anim Sci 27(4):512-517.
- 4. Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 75-30 June 76.
- 5. Schmidt, L.H. 1973 Infections with Plasmodium falciparum and Plasmodium Vivax in the Owl Monkey Model Systems for Basic Biological and Chemotherapeutic Studies. Trans Roy Soc Trop Med Hyg 67(4):446-473.
- 6. Brumback, R.H. and Willenborg, D.O. 1973. Serotaxonomy of Aotus. A Preliminary Report. Folia Primat 201:106-111.
- 7. Collins, W.E., Stanfill, P.S., Skinner, J.C., et. al. 1974.

  Studies on Human Malaria in Aotus Monkeys, IV. Development of Plasmodium falciparum in two Subspecies of Aotus trivirgatus.

  J. of Parasit 60(2):355-358.
- 8. Wellde, B.T., Johnson, A.J., Williams, J.S. et. al. 1971.
  Hematologic, Biochemical, and Parasitologic Parameters of the
  Night Monkey (Aotus trivirgatus). Lab Anim Sci 21(4)575-580.
- 9. Hunt, R.D., Van Zwieten, M.J., Baggs, R.B. et. al. 1976. Glomerulonephritis in the Owl Monkey (<u>Aotus trivirgatus</u>). Lab Anim Sci 26(6):1088-1092.

Table 1: Selected reproductive data for 31 pairs of owl monkeys over a 24 month period

	Successful Breeding pairs	Unsuccessful Breeding pairs	Total
Non-conceiving females	NA <sup>3</sup>	9	9
Conceiving females	17	5	22
Conceptions <sup>2</sup>	23	16	39
Births	21	0	21
Abortions	0	2	2
Resorptions	2	14	16

<sup>1.</sup> Successful pairs are only those that produced viable offspring.

<sup>2.</sup> Conceptions were detected by urine pregnancy test and/or palpation.

<sup>3.</sup> Not applicable.

Table 2: Karyotypes of 253 Aotus by geographic origin and sex.

Karyotype designation	Diploid (2n) chromosome number	Presumed country of origin	Number of males	Number of females
I	54	Brazil	18	11
11	54	Colombia	44	43
III	53	Colombia	41	47
IV	52	Colombia	13	14
٧	46	Colombia	2	4
VI	49 males 50 females	Bolivia	8	6
VII	52	Peru	0	1
Hybrid	49	(V X III)	1	0

Table 3: Incidence of conceptions, births and abortions or resorptions in 31 pairs of owl monkeys in 24 months by karyotype

M	Karyo lale &	oty Fe	pe male	No. Pairs	Conceiving females	No. conceptions	Live births	Abortions resorptions
Group A <sup>2</sup>	I	х	II	1	1	1	1	0
	I	X	III	1	1	1	1	0
				2	2	2	2	0
Group B <sup>3</sup>	II	χ	II	5	5	8	6	2
	II	X	IV	1	0	0	0	0
	IV	X	II	4_	3	7	2	_5_
				10	8	15	8	7
Group C <sup>4</sup>	II	X	111	4	1	3	0	3
	III		II	5	4	5	5	0
	III	X	IV	2	2	7	0	7
	IV	X	III	1_	1	1	1	0
				12	8	16	6	10
Group D <sup>5</sup>	III	X	III	7	4	6	5	1

Conceptions were detected by urine pregnancy test and/or palpation.
 Group A - Pairs with dissimilar types of chromosomes.
 Group B - Pairs with compatible chromosomes.
 Group C - Pairs with one hybrid (karyotype III) parent
 Group D - Pairs with both hybrid (karyotype III) parents.

Table 4: Fertility and parity of 62 owl monkeys by age groups and sex during a 24 month period.

	Males		Females				
Estimate age 1	Total	fertile (%) <sup>2</sup>	Total	fertile (%) <sup>2</sup>	Parous (%) <sup>3</sup>		
Young adult	16	11(69)	19	15(79)	10(53)		
Mature	6	5(83)	8	3(38)	3(38)		
Fully mature	8	6(75)	3	3(100)	3(100)		
01d	1	0(0)	1	1(100)	1(100)		

- Age estimated by dental wear: Young adults had sharp points on permanent canine and molar teeth. Mature monkeys had rounded points on canines and molars. Fully mature monkeys had molars with flattened occlusal surfaces and old monkeys had teeth that were worn to within a few millimeters of the gingiva.
- 2. Fertility was demonstrated by conception in the female as shown by palpation or urine pregnancy test.
- Parity was demonstrated by producing a live offspring during the study period.

Table 5: The body weights of productive and nonproductive breeder owl monkeys.

		Body w	eight in gm
	Number	Mean	Range
Productive breeder	s <sup>1</sup>		
males	17	877	746-1013
females	17	870	671-1077
Nonproductive bree	ders		
males	14	906	797-1034
females	14	864	778-979

<sup>1.</sup> Produced live offspring.

Qualitative assays for urinary chorionic gonadotropin from 31 pairs of owl monkeys Table 6:

No. of animals Parous females <sup>4</sup> 5 Fertile/nonparous females <sup>4</sup> 5 Nonfertile females <sup>5</sup> 9	No. of tests 888 376 424	Correct (%) 790 (89) 326 (87) 375 (88)	1 0 1 .		Equivocal (±) in non-pregnant females 43 (4.8) 23 (6.1) 34 (8.0)
	1688	1491 (88)	39 (2.3)	58 (2.3)	100 (5.9)

Sub-Human Primate Pregnancy Test kit (Ortho Diagnostics, Inc., Rarital, NJ).

Equivocal responses during pregnancy were considered positive (correct) responses.

Females which gave birth to live offspring during the 22-24 month study period.

Females which conceived but pregnancies terminated in abortion or resorption and no births occurred during the study period.

Females which never conceived during the study period. 5

Not applicable.

Table 7: Mean eruption time for deciduous teeth of 28 Aotus monkeys

Too	oth	Mean	Age in Weeks 1 Standard deviation		% of times observed erupting first	
I <sub>1</sub>	MX <sup>2</sup>	2.3	0.7	1.0 - 3.5	26	
	MD <sup>3</sup>	2.3	0.8	0.5 - 3.5	26	
I <sub>2</sub>	MX	3.5	0.9	1.5 - 4.5	0	
	MD	2.9	0.8	1.0 - 4.5	70	
С	MX	4.7	1.0	1.5 - 6.0	27	
	MD	4.6	1.2	2.0 - 6.5	23	
Pı	MX	3.5	0.7	1.5 - 5.0	13	
	MD	3.4	0.7	2.0 - 5.0	27	
P <sub>2</sub>	MX	3.8	0.8	2.0 - 5.5	21	
	MD	3.8	0.9	2.0 - 5.0	18	
P <sub>3</sub>	MX	5.6	1.2	3.0 - 8.0	13	
	MD	5.5	1.1	3.0 - 7.5	27	

<sup>1.</sup> Percentage of observations in which the maxillary or mandibular tooth erupted before its ipsilateral counterpart. The difference between the sum of MX + MD and 100% equals the percentage of observations in which the maxillary and mandibular teeth erupted together.

<sup>2.</sup> MX = Maxillary tooth

<sup>3.</sup> MD = Mandibular tooth

Table 8: Mean eruption time for permanent teeth of Aotus monkeys

Too		No. of Observations	Mean	Age(Months) 1 Standard deviation	Range	% of times observed erupting first <sup>2</sup>
I	MX 3	24	9.4	0.8	8.0 - 11.0	55
	MD <sup>4</sup>	18	9.6	0.6	9.5 - 11.0	9
I <sub>2</sub>	MX	13	10.8	0.6	10.0 - 12.0	64
	MD	8	10.6	0.4	10.0 - 11.0	0
С	MX	18	15.0	1.3	13.5 - 18.0	29
	MD	15	14.0	1.0	12.5 - 16.0	43
P <sub>1</sub>	MX	12	12.4	1.1	11.0 - 14.5	33
	MD	13	12.4	0.9	11.5 - 14.0	17
P 2	MX	12	11.9	1.0	10.5 - 14.0	67
_	MD	13	12.3	0.8	11.5 - 14.0	0
P <sub>3</sub>	MX	12	11.5.	0.9	10.0 - 13.0	13
	MD	16	11.0	0.7	10.0 - 12.0	50
M <sub>1</sub>	MX	36	4.9	0.6	4.0 - 6.0	0
	MD	38	4.3	0.5	3.5 - 5.0	84
M <sub>2</sub>	MX	28	7.3	0.6	6.5 - 8.5	0
	MD	32	6.4	0.8	5.0 - 7.5	100
M <sub>3</sub>	MX	17	11.2	1.1	10.0 - 13.0	0
	MD	18	9.9	1.2	8.5 - 11.5	91

<sup>1.</sup> Observation is the eruption of a single tooth

<sup>2.</sup> Percentage of observations in which the maxillary or mandibular tooth erupted before its ipsilateral counterpart. The difference between the sum of MX + MD and 100% equals the percentage of observations in which the maxillary and mandibular teeth erupted together.

<sup>3.</sup> MX = Maxillary tooth

<sup>4.</sup> MD = Mandibular tooth

Table 9: Incidence of single fast and slow moving albumin, double albumin, and triple alpha globulins observed by serum protein electrophoresis in 156 owl monkeys by karyotype.

			Monkeys having						
Karyotype	Monkeys Sex examined  Male 13 Fem. 8 Both 21		Single fast albumin (%)	Single slow albumin (%)	Double	Triple alpha globulin (%)  8 7 15 (71)			
I			13 8 21 (100)	0 0	0 0				
II	Male Fem Both	34 30 64	19 17 36 (56)	$\frac{5}{1}$ (9)	10 12 22 (34)	3 6 9 (14)			
III	Male Fem. Both	28 27 55	13 14 27 (49)	$\frac{3}{1}$ (7)	12 12 24 (44)	4 3 7 (13)			
IV	Male Fem. Both	6 6 12	$\frac{\frac{3}{3}}{6}$ (50)	1 1 2 (17)	2 2 4 (33)	0 3 3 (25)			
V	Male 0 Fem. 1		ND <sup>1</sup> O	ND O	ND 1	ND O			
VI	VI Male 3 Fem. 0		O ND	3 ND	O ND	2 ND			

<sup>1.</sup> No animal examined

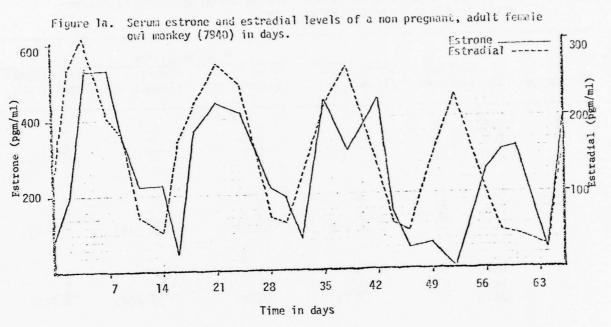
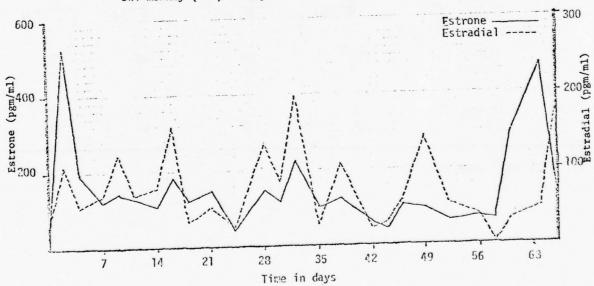
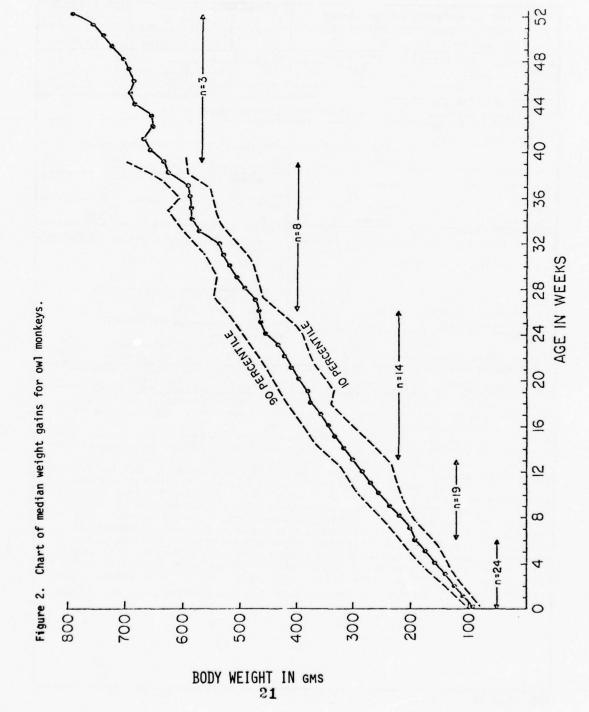


Figure 1b. Serum estrone and estradial levels of a non pregnant, adult female owl monkey (126) in days.





RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY					DA OB 6512		77 09 30			CONTROL SYMBOL	
76 10 01	K. Completion	S. SUMMARY SCTYS	E. WORK SECURITY	NA			B'N INSTR'N L	ON SPECIFIC	DATA- R ACCESS	A WORK UNIT	
10. NO./CODES:*	PROGRAM ELEMENT	PROJECT	NUMBER	TASK A	REA NUMB	ER		WORK UNI			
& PRIMARY	61101A	3A161101A	191C	00			120				
b. CONTRIBUTING											
c. CONTRIBUTING											
	Security Classification Code		1 11-11 -6 1								
	CHHOLOGICAL AREAS®	or the cer	wall of	Neiss	eria m	enı	ngitiai	.S			
010100 Mici											
13. START DATE	Oblology	14. ESTIMATED COM	PLETION DATE	TIS FUNC	ING AGENCY			Tie procom	ANCE MET	400	
72 07		77 09		DA	1		1	C. In-House			
17. CONTRACT/GRANT		17 09		-	OURCES EST		1				
& DATES/EFFECTIVE:	NA	EXPIRATION:		IS. RES	PRECEDING	MATE	PROFES	HONAL MAN YR	S & FUN	DS (In thousands)	
b. NUMBER:*				FISCAL	76		3		202		
C TYPE:		& AMOUNT:		YEAR	CURRENT		+		+-		
& KIND OF AWARD:		f. CUM. AMT.			77		4		2	16	
19. RESPONSIBLE DOD	DRGANIZATION			20. PERF	ORMING ORG	ANIZA	TION			T	
NAME: Walte	er Reed Army I	nstitute d	f Research						ute of	Researc	
							of CD&I				
ADDRESS: Washi	ington, DC 20	0012		ADDRESS	· Wash	ing	ton, DO	20012			
							(Pumlah 38AH		[netfution]		
RESPONSIBLE INDIVIDU				0.0000000000000000000000000000000000000				B. Ph.	D.		
	nd, Garrison,	COL		TELEP	HONE: 202	-5/	6-3758				
TELEPHONE: 202-	-576-3551				SECURITY A						
21. GENERAL USE				The second	E INVESTIG			D D	1.		
Foreign int	elligence not	considere	ed	NAME:			-	B. Bran		DA	
	BACH with Security Classific			MAME:	Prote	in:	(11) 13	l. Schne	acchai	ride:	
	charide; (U)								acciiai	Tuc,	
	IVE, 24 APPROACH, 28							locarity Classific	etten Code.		
	nalyze the cel					ngo	coccus	with em	phasis	s on	
extracting a	and purifying	the domina	ant antigen	s. T	he goa	1 i	s to de	evelop c	andida	ate	
	r this disease										
	ein, lipopolys										
biophysical	ly and immunoc	chemically.	Antibody	resp	onses	of	animals	and hu	mans v	will be	
	a variety of										
absorbent te	echniques. Ce	ellular imm	nune respon	ses w	ill be	me	asured	by in v	itro	technique	
25 (U) 76 10	0 - 77 09 Ser	isitivity t	o meningoc	occal	intec	110	n in ot	nerwise	resis	stant	
individuals	may be due to	the prese	ence of IgA	whic	n is c	apa	pre of	DIOCKIN	g the	comple-	
ment mediate	ed bacteriolyt	tic activit	y of anti-	menin	gococc	aı	igG and	I Igm.	A new	group b	
	al vaccine, co										
	r membrane pro										
	to inhibit hu								The no		
errective pr	rocedure for s	ity agains	group A me	citiv	e Neie	was	in ic	lirected			
	ricidal activi		continue un								
LPS antigens										1 report	
see walter K	Reed Army Inst	itute of R	esearch Ann	nual	Progres	SS	Report,	1 Jul	76 - 3	0 Sep 77	

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 120 Antigenic components of the cell wall of  $\underbrace{\text{Neisseria}}_{\text{meningitidis}}$ 

Investigators.

Principal: Samuel B. Formal, Ph.D.

Wendell D. Zollinger, Ph.D.; LTC J. McLeod Griffiss, MC,MD Associate: Herman Schneider, Ph.D.; Brenda Brandt; Robert Mandrell; SSG Dennis Broud; SP5 Lawanna Sachse; SP4 Glynne Williams

1. Studies on the specificity of naturally acquired human bactericidal antibodies to group B meningococci have led to the development of a new group B meningococcal vaccine which has substantially increased antigenic activity and has been shown to be safe and immunogenic in animals.

- 2. The existence of group A meningococcal sub-types which correlate with geographical origin was demonstrated by a new serotyping procedure based on the LPS antigens.
- 3. Susceptibility to meningococcal disease in otherwise immune individuals may be due to the presence of IgA which is capable of blocking the complement mediated bacteriolytic activity of antimeningococcal IgG and IgM.
- 4. Capsular polysaccharides from group A and group C meningococci have been shown to be safe and effective vaccines, but group B capsular polysaccharide has thus far been non-immunogenic in human beings. Group B meningococci, however, have amply demonstrated their capacity to cause epidemic meningococcal disease as evidenced by the epidemic among military basic recruits between 1964 and 1967 and the more recent outbreaks in Europe and among restricted civilian populations in this country.

Over the past 8-10 years at least four different group B vaccine studies have been conducted by this department. In table 1, these results are summarized and compared to the groups A and C capsular polysaccharide vaccines. Although the vaccines have all proven to be safe in animals and in volunteers, it is evident that a poor correlation exists between the immunogenicity of these vaccines in animals and in human beings. Although the A and C capsular polysaccharides were not immunogenic in animals, they proved to be very effective immunogens in human beings. On the other hand, the group B capsular polysaccharide lacked immunogenicity in both animals and man, and the type 2 outer membrane protein vaccines were immunogenic in animals but did not induce bactericidal antibodies in man. These results demonstrate the inadequacy of animal models for estimating immunogenicity of meningococcal antigens in man.

The most recent vaccine tested (type 2 protein plus group C polysaccharide) was partially successful in that increased levels

of bactericidal antibodies to group B meningococci were induced. These antibodies, however, were group specific and directed against the group B polysaccharides rather than type 2 protein as anticipated. Although the immunological mechanism by which this bactericidal antibody to the group B capsular polysaccharide was induced is not understood, these studies demonstrated that bactericidal antibody to the group B polysaccharide can be induced in human beings. It was also evident that the methods used to prepare the type 2 outer membrane protein component of the vaccine produced a product which was capable of inducing type-specific bactericidal antibodies in rabbits, but not in man.

A good correlation between the presence of serum bactericidal antibodies and immunity to the meningococcus has been demonstrated. The prevalence and antigenic specificity of human antibodies with bactericidal activity toward group B meningococci was investigated. Normal human sera obtained from 134 incoming Army recruits were assayed for the presence of antibodies against group B type 2 meningococci. Bactericidal antibodies with a geometric mean titer of 1:300 were detected in 89% of these sera. Twenty-eight of these sera were further analyzed to determine the specificity of the bactericidal antibodies. It was found that inhibition with group B capsular polysaccharide or absorption with a group B strain of a completely different serotype resulted in loss of bactericidal activity in all 28 sera. Absorption with a group C strain that shared the type 2 protein antigen and the type 3 LPS antigen, however, did not remove the bactericidal activity from any of the sera. These results indicated that the bactericidal antibodies in these normal sera were directed against the group B capsular polysaccharides. Of six sera from laboratory personnel all had bactericidal activity, and in five of these the bactericidal antibodies were also directed against the B polysaccharide. In the one remaining serum, bactericidal antibodies against the type 2 protein as well as the B polysaccharide were present.

Convalescent sera from patients with disseminated meningo-coccal disease caused by type 2 (group B and group C) meningococci were also analyzed. Of 7 sera from group B cases and 11 from group C cases all were bactericidal to the group B type 2 strain 99M. Absorption of these sera with a group B strain of unrelated sero-type removed the bactericidal activity from 8 of the 18 sera indicating the presence of bactericidal antibodies to the B polysaccharide and the absence of type 2 - specific bactericidal antibodies in these 8 sera. The remaining ten sera contained type-specific bactericidal antibodies which were predominantly directed against the serotype protein antigens. The precise specificity of these antibodies is still under investigation.

Thus, bactericidal antibodies with specificity for the group B polysaccharide and for the serotype protein antigens have been identified. Of a total of 34 normal sera with bactericidal activity

only one had detectable bactericidal antibodies against the serotype proteins while 10 of 18 convalescent sera had type-specific bactericidal antibodies.

In the absence of a suitable animal model in which to test potential vaccines for antigenicity and immunogenicity, the capacity of a group B antigen to inhibit a standard amount of human bactericidal antibodies has been used as an index of antigenic activity. Studies with the group B capsular polysaccharides have shown a strong correlation between molecular size and antigenic activity. In our initial efforts to determine the specificity of human antibodies with bactericidal activity toward group B meningococci, we found that many sera had group B specific bactericidal activity which was inhibited by some preparations of group B capsular polysaccharide but not by others. Subsequent experiments revealed that antigenic activity correlated best with the molecular size of the preparations. Group B polysaccharide is known to be extremely susceptible to acid hydrolysis. Incubation overnight at pH 5 and 37°C results in extensive degradation of the polysaccharide. The relationship between molecular size and antigenic activity was investigated in more detail by performing a hydrolysis time curve on intrinsically labeled <sup>3</sup>H-group B polysaccharide (<sup>3</sup>H-Bsss). High molecular weight <sup>3</sup>H-Bsss was incubated at 37°C in 0.1N ammonium acetate buffer pH 5.0 for different periods of time after which the samples were frozen, lyophilized and re-dissolved in 0.01 N tris-C1 pH 7.5. The resulting samples were assayed for antigenic activity in four different Bsss-specific serologic assays: inhibition of human bactericidal antibodies, inhibition of human hemagglutinating (HA) antibodies, binding to hyperimmune horse anti-Bsss antibodies in a Farr assay and formation of a precipitin line with these antibodies in immunodiffusion tests. The molecular size of the samples was estimated by relative mobility on sodium dodecylsulfate polyacrylamide gels using globular proteins as molecular size markers, and the amount of terminal or free sialic residues was determined by reactivity in the thiobarbituric acid assay for sialic acid. The results of these assays are summarized in figure 1. The capacity of the <sup>3</sup>H-Bsss to inhibit the human bactericidal and HA antibodies decreased sharply with decreased molecular size, but the percentage of the 3H-Bsss that could bind to antibody in the Farr assay decreased only gradually. Thus, after hydrolysis for 4 hours when the mean apparent molecular weight had fallen to about 10,000 daltons, inhibitory activity in the bactericidal and HA assays was less than 1% of the original activity while in the Farr assay 70% of the antigen was still capable of binding to antibody. The four and six-hour hydrolysis samples were also still able to form precipitin lines with the hyperimmune horse serum. These results suggest the presence of two different antigenic determinants on the B polysaccharide. One determinant, which reacts with human bactericidal antibodies, appears to be found only on molecules of high molecular weight whereas the other determinant, probably associated with the main chain of the polysaccharide, is present on both high and low molecular weight

molecules. Since very similar results were obtained with group C polysaccharide, these properties are not unique to the B polysaccharide. The group C polysaccharide, however, is much more resistant to acid hydrolysis.

The question arises as to whether the determinant associated with high molecular weight polysaccharide is actually located on the polysaccharide itself or whether it might be formed by the binding of the polysaccharide to a non-polysaccharide moiety which is dissociated or inactivated by mild acid hydrolysis. We have investigated this question using two approaches. The first was to determine if the polysaccharide remained active following molecular sieve chromatography in the presence of 1% sodium deoxycholate (DOC). Partially purified Bsss was chromatographed on Sepharose CL-4B in 0.05 M tris-Cl pH 7.4 or in 1% DOC, 0.05 M Glycine, 0.005 M EDTA, pH 8.8. Exposure to the detergent caused the largest polysaccharide to be reduced in size presumably as a result of disaggregation or dissociation from a non-polysaccharide moiety. The polysaccharide recovered from the detergent, however, retained its capacity to react with human bactericidal antibodies. a second approach, high molecular weight Bsss was banded on isopycnic CsCl density gradients. The polysaccharide produced a single asymetric peak at a density of 1.68 gm/cc. Again, the peak fractions retained the capacity to react with human bactericidal antibody. Although further studies are needed, these results suggest that the "high molecular weight determinant" is located on the polysaccharide itself rather than on some other moleculer non-covalently bound to it. The determinant may be dependent on a particular conformation of the polysaccharide which can occur only when the molecule is sufficiently large.

The failure of the previous meningococcal protein vaccines to induce type-specific bactericidal antibodies in man after they had been shown to induce such antibodies in rabbits indicated the need for a better understanding of the protein antigens against which human bactericidal antibodies are made as a result of natural meningococcal infections. Two convalescent sera from patients with group B, type P2 disseminated meningococcal disease that were identified as having type-specific bactericidal antibodies were absorbed as necessary to remove antibodies not directed against the protein antigens. A number of different antigen preparations were then tested for their capacity to inhibit the bactericidal activity of these sera toward type 2 meningococci. Outer membrane complex, (OMC), consisting principally of proteins, lipopolysaccharide (LPS) and phospholipid; inhibited down to a concentration of about 10 µg protein/ml whereas purified capsular polysaccharides or LPS were not able to inhibit at concentrations up to 500 µg/ml. Although pronase treatment of the OMC destroyed or greatly reduced its capacity to inhibit, the isolated protein component of the OMC had no activity at concentrations up to 500 µg/ml. These results suggested that the isolation procedure, which was the same as that used to prepare the previous protein vaccine and involved exposure of the protein to extremes of pH, caused the protein to lose its capacity to react with human bactericidal antibodies. The capacity of the protein to react with rabbit bactericidal antibodies, however, remained intact.

Further studies were conducted to determine the factors affecting the antigenic activity of the serotype proteins. A normal human serum containing type-specific bactericidal antibodies was absorbed with strain 99M capsular polysaccharide and LPS to remove antibodies to non-protein antigens. The type-specificity of this serum was demonstrated by the failure of OMC from seven different non-type 2 strains (4 group B and 3 group C) to inhibit its killing of the type 2 strain 99M. OMC from another group B type 2 strain M986, however, inhibited strongly. Inhibition of the bactericidal activity of this serum toward strain 99M was then used as a measure of antigenic activity of 99M OMC and its components following a variety of different treatments (table 2). Purified LPS and group B polysaccharide were unable to inhibit at 500 µg/ml. Treatment of the OMC with pronase increased the concentration required for 50% inhibition (MIC $_{50}$ ) from 3.3 to >800 µg protein/ml which demonstrates the protein nature of the determinant. Each major step in the current procedure for separating the protein and LPS components of the OMC was tested for its effect on the antigenic activity of the protein. Precipitation and washing of the OMC with ethanol (80%) resulted in a small increase of the  ${\rm MIC}_{50}$  from 3.3 to 5.9  $\mu g$  protein/ml. The OMC was dissociated in 3% DOC, 0.01 M EDTA, 0.05 M glycine pH 8.8 and chromatographed on a column of Sephadex G 100 in 1% DOC, 0.005 M EDTA, 0.05 M glycine pH 8.8. When all fractions between the void volume and the total volume of the column were pooled and the antigen recovered by precipitation and washing with ethanol, the  ${
m MIC}_{50}$  of the resulting material was 12. If the protein and the LPS peaks were pooled and precipitated separately and tested, the  ${
m MIC}_{50}$ rose to 78 µg protein/ml for the protein and 210 µg LPS/ml for the LPS peak. Mixing of the LPS fraction with the protein fraction after removal of the DOC by ethanol precipitation and washing resulted in about the same MIC<sub>50</sub> as the protein alone. These results suggest that a binding or interaction between the protein and the LPS (and/or phospholipid) is required for full antigenic activity of the type 2 protein antigen. Although some antigenic activity was observed with the protein fraction alone 95% of the activity of the native antigen (OMC) was lost in the process of separating it from the LPS and lipid. Indeed, the residual activity of the protein may result from interaction with a small amount of contaminating LPS and/or lipid.

Between September and December 1976 two new candidate vaccines for use against group B meningococci were prepared in conjunction with the Department of Biologics Research, WRAIR. These two lots, designated Bsss-P2 WZ-2 and Bsss-P2 PA-3, have been characterized with respect to composition, antigenic activity and safety in animals, and it is anticipated that tests in a small number of human volunteers will be completed by January, 1978. Both lots are derived from the group B type P2 strain B11 and consist principally of high molecular weight capsular polysaccharide and outer membrane protein. Lot WZ-1 has a protein: polysaccharide ratio of 1:1 while in lot PA-3 the ratio is

2:3 (table 3). The proteins present were shown by sodium dodecyl sulfate polyacrylamide gel electrophoresis to be essentially the same in both lots and to correspond to the major outer membrane proteins of the organism. Lot WZ-2 is slightly cleaner with respect to contaminating nucleic acid and LPS (as judged by pyrogenicity tests), but both lots passed required toxicity and pyrogenicity tests at the levels established by the Bureau of Biologics for the meningococcal groups A and C polysaccharide vaccines.

The antibody response of rabbits to the vaccines is summarized in table 4. The rabbits were given two 50  $\mu g$  doses (based on protein) either intravenously or subcutaneously three weeks apart. Sera drawn at 0 and 5 weeks were assayed for antibodies using three different assays. The antibody responses to the two lots were very similar. Bactericidal antibodies against the vaccine strain increased over 50-fold, antibodies to the group B polysaccharide increased approximately 2-fold and antibodies to the OMC which contains both serotype protein and LPS antigens increased several hundred fold. Most of the latter antibodies were likely directed against the serotype protein, since only a low level of antibodies against the purified LPS was detected. The antibody response in rabbits was therefore directed primarily against the serotype protein antigens.

Although the strong antibody response of rabbits to the protein component of the vaccines demonstrates the presence of antigenically active serotype protein in the vaccine, past experience indicates that this does not mean that the vaccines will produce a similar response in man. A second and probably more relevant test of antigenic activity was also conducted. Human sera with bactericidal antibodies specific for either the group B polysaccharide or the serotype 2 protein were inhibited by different concentrations of the vaccines and several control antigens (table 5). The B polysaccharide component of the vaccine was twice as active as the best purified B polysaccharide preparation (lot 3XCA) that has been tested in man and 4 times as active as the antigenically identical E. coli Kl capsular polysaccharide included as a control for antibody specificity. The protein component of Lot PA-3 which was not subjected to gel filtration in the presence of sodium deoxycholate was considerably more active in inhibiting human type 2-specific bactericidal antibodies than Lot WZ-2 which was exposed to detergent during its preparation. As pointed out above, previous protein vaccine lots were not able to inhibit human type-specific bactericidal antibodies. Thus, the present vaccine lots are significantly more active than previous trial vaccine lots as judged by their ability to interact with human bactericidal antibodies and may therefore elicit a better antibody response in man.

The presence of both outer membrane protein and capsular polysaccharide in the vaccines raises the question of whether there is an interaction or binding between the two. If the polysaccharide is bound to the protein, the latter may act as a carrier for the polysaccharide and thereby increase its immunogenicity. Evidence for binding between the protein and polysaccharide components was obtained in two ways. Gel filtration of the vaccines on Sepharose CL-4B in the presence of detergent (1% sodium deoxycholate) resulted in a decrease in apparent size of part of both the polysaccharide and the protein as compared to their behavior in the absence of detergent. This effect was reversible, since removal of the detergent by ethanol precipitation and washing resulted in the same elution profile in the absence of detergent as before.

Secondly, analysis of the vaccines by equilibrium density gradient centrifugation in CsCl revealed that part of the polysaccharide banded at a density of  $1.66~\rm g/cc$  which is the density where purified group B polysaccharide bands, but part of the polysaccharide banded at or near the top of the gradient (about  $1.45-1.5~\rm g/cc$ ) where the bulk of the protein banded. This result clearly demonstrates binding between the protein and the polysaccharide. This binding increases the solubility of the protein and may increase the immunogenicity of the polysaccharide.

2. Serotyping of group A meningococci using the bactericidal or immuno-diffusion methodologies developed for serotyping of groups B and C meningococci has been largely unsuccessful. This is due partly to the difficulty of finding a complement source without intrinsic bactericidal activity toward group A meningococci and partly due to the presence of a relatively dominant outer membrane protein antigen which appears to be shared by all group A strains and tends to mask other type-specific determinants which may be present.

We recently described a procedure for serotyping of meningococci based specifically on either the protein or the lipopolysaccharide (LPS) antigens of the outer membrane. This procedure involves inhibition of a solid phase radioimmunoassay (SPRIA) by outer membrane complex (OMC) extracted from the strain to be typed. Using the SPRIA inhibition method and rabbit antisera, group A strains of N. meningitidis have been successfully serotyped on the basis of both their LPS and outer membrane protein antigens. Initial studies using typing sera prepared with group B and group C strains indicated that serotype antigens on group A strains were mostly different from those on strains of groups B, C and Y. Only 15-20% of group A strains had any of the antigens that had been identified on non-group A strains. As a consequence, new typing sera were prepared using group A strains. These sera were used to identify 3 different LPS antigens and 5 different protein antigens among group A strains. LPS serotyping employed reactions between purified LPS as the solid phase antigen and unabsorbed homologous antisera. The protein typing, on the other hand, employed OMC as the solid phase antigen and homologous antiserum absorbed to remove antibodies to LPS, group A polysaccharide, and cross-reactive protein determinants.

About 80 group A strains obtained from several parts of the world including Canada, Finland, Brazil, West Germany, Africa and the U.S.A. (pacific northwest) were serotyped and the results correlated with geographical origin. In the LPS system 96% of the strains were typable

and in the protein system 84% were typable. A correlation between geographical origin and LPS serotype but not protein serotype was evident. For example, 4 of 5 strains from Finland were LPS type 11, all 5 from West Africa were LPS type 9, 9 of 10 from West Germany were LPS type 10 and 40 of 45 from the Pacific Northwest were LPS type 10. It thus appears that the LPS serotype may be a useful epidemiological tool for the study of group A meningococcal disease.

The significance of the group A protein serotype antigens and their relationship to those on groups B and C meningococci is still under investigation.

3. Previous work in the Department of Bacterial Diseases correlated susceptibility to systemic meningococcal disease with the absence of strain-specific bactericidal activity normally present in the serum of 80-90% of young adults. Circulating anti-meningococcal IgA was subsequently shown to be capable of blocking the complement-mediated bacteriolytic activity of anti-meningococcal IgG and IgM. In addition, levels of circulating anti-meningococcal IgA sufficient to completely abrogate immune lysis of the infecting meningococcus have been demonstrated in the sera of military recruits early in the course of invasive disease due to serogroups B, C and Y N. meningitidis and before the development of whole-serum bactericidal activity. These data led to speculation that acquisition of specific, blocking IgA may be a factor in the sudden emergence of epidemic susceptibility to the meningococcus in sub-populations at greater than expected risk of disease.

The critical role of gut-associated lymphoid tissue (GALT) in the IgA immune system suggested that IgA might be derived from stimulation of the GALT by enteric bacteria of differing genera which elaborate surface antigens cross-reactive with those of the meningococcus. If so, then such enterically carried, cross-reacting organisms should be present in a population undergoing epidemic meningococcal disease.

An opportunity to test this assumption was provided by an outbreak of group A meningococcal disease in the Pacific Northwest. Beginning in June, 1975, and continuing through 1976, an outbreak of disseminated group A meningococcal disease occurred in four Pacific Northwest cities: Portland, OR, Seattle, WA, Vancouver, B.C. and Anchorage, AL - the first such outbreak of group A meningococal disease in the U.S. in thirty years. The most striking epidemiological aspects of the outbreak was its focal nature. Sixteen (table 6) of the first seventeen cases in Portland and Seattle, from which data were available, occurred in residents or habitués of Skid Road areas. Fifteen were adults, and fourteen alcoholics. With the exception of one child in Portland, there was no spill-over of cases into non skid-road areas.

Since immunity to the meningococcus is normally well developed among adults, we initiated a serological survey of the at-risk population to determine the prevalence of immunity. Table 7 lists

the populations surveyed and their designation. Of 235 sera submitted by the University of Washington, 75 were obtained from Seattle skid-road inhabitants 6 months to a year prior to the outbreak of meningo-coccal disease, 50 were from non-alcoholic American Indians attending out-patient facilities at the public health hospital and 108 were from 3 Alcoholic Treatment Centers within the Seattle area.

Of 112 sera submitted by the CDC, 44 represented a random sample of skid-road inhabitants in Seattle, whose age, sex and ethnic background did not significantly differ from the cases in that city. Thirty four sera were from a tangential population in Seattle, who were awaiting pre-employment physical examinations at a facility in proximity to skid-road; designated non skid-road controls, their socio-economic status was mid-way between casually employed skid-roaders and stably employed blue-collar workers. An additional 14 sera were from case contacts, and the remainder from other non-random categories.

Finally, 148 sera from incoming recruits at Ft. Ord, CA, in January, 1975, were used to sample the general level of immunity among adults from the Western U.S.

The 435 sera were assyed for antibody to the group A-specific capsular polysaccharide using a Radioactive Antigen Binding Assay. Data are expressed as ngm antigen bound by 50 microliters of sera. For this assay, 10 ngm antigen bound is equivalent to 3.5 mcgm/Ab/ml sera. The Kolmogorov-Smirnov two-sample test, a non-parametric method, was used to determine the significance of differences in frequency of anti-meningococcal polysaccharide antibody among the populations surveyed.

Surprisingly, the prevalence of immunity to the group A meningo-coccal capsule was found to be significantly higher among the Pacific Northwest populations than among the recruits (figure 2). That this was the result of a specific immunizing event and not a reflection of the hyper-globulinemia which might be expected among alcoholics, was shown by comparing immunity to the chemically unrelated group C capsular polysaccharide (figure 2).

There were no differences in prevalence of antibody to the group A capsule between sera submitted by the CDC, and those submitted by the University of Washington, nor between sera obtained prior to the outbreak and sera obtained during it (figure 3). This last suggests that the immunizing event occured among the at-risk population prior to significant aquisition of the group A meningococcus.

When the Pacific Northwest sera are broken down into sub-populations (figure 4), it is evident that the population with the highest level of immunity was precisely that at greatest risk. Non-alcoholic Indians and the CDC non skid-road control population did not differ significantly from recruits, while alcoholics and skid-road inhabitants did. The highest prevalence was among patients at the three Alcoholic Treatment

Centers. Alcoholism alone, however, could not be incriminated, for the prevalence of immunity among treatment staff at the Centers did not differ from that among patients. Nor was ethnic background a factor: as can be seen in the figure 5 which compares the geometric mean ngm antigen bound and 95% confidence interval for each population; alcoholic Indians (AI) were more immune than non-alcoholic Indians, (IHBC) but were not different from alcoholic non-Indians (NI).

Among alcoholics, differences were seen in the prevalence of antibody at each of the three treatment centers. Convalescent Indian alcoholics, designated as "dry" and housed in a suburban facility were less immune than acute Indian alcoholics seen at a treatment center in skid-road itself, and designated as "wet". Alcoholics of all races, seen at Harborview, (HV) situated on a hill above skid-road, were intermediate, suggesting that intimacy with the skid-road culture was the most important determinant of level of immunity.

To determine if organisms other than the group A meningococcus could have stimulated the high levels of antibody observed, throat and rectal cultures were obtained from the 112 individuals, comprising the CDC group, and grown on Mueller-Hinton Agar containing equine antibody to the group A polysaccharide. In addition, rectal smears were tested with fluorescein-conjugated anti-group A Ab.

Five individuals from both Seattle and Portland, carried enteric flora which elaborated the group A capsular polysaccharide-mannosamine phosphate (table 8). Two strains of <a href="Str. faecalis">Str. faecalis</a> and one of <a href="Bacillus pumilis">Bacillus pumilis</a> were recovered, while 2 faecal smears were positive for cocci by flourescent antibody only. Significantly, the carriage rate of cross-reactive enteric flora was the same as the carriage rate of the group A meningococcus among skid-road controls in Seattle (4.5%), while 14% of case contacts were positive for enteric carriage, including the father of the child in Portland who was not associated with skid-road. Pharyngeal carriage of the group A meningococcus was higher among alcoholics in Seattle, than among the random sample of skid-road inhabitants.

In summary, a focal outbreak of group A meningococcal disease occurred among skid-road inhabitants of 4 Pacific Northwest cities. Paradoxically, the population at greatest risk possessed significant and prior immunity to the causative organism, which appeared to be environmentally related, and were colonized by cross-reactive enteric organisms as well as the group A meningococcus. This immunologic paradox may be related to the temporal kinetics of co-colonizations with organisms stimulating differing sites of the immune system, resulting in an imbalance in the stimulation of IgA and lytic IgM and IgG, with resulting susceptibility.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 120 Antigenic components of the cell wall of Neisseria meningitidis

### Literature Cited.

#### References:

- 1. Zollinger, W. D., Dalrymple, J. M., Artenstein, M. S. Analysis of parameters affecting the solid phase radioimmunoassay quantitation of antibody to meningococcal antigens. J. Immunol. 117:1788-1798, 1976.
- 2. Mandrell, R. E., Zollinger, W. D. Lipopolysaccharide serotyping of Neisseria meningitidis by hemagglutination inhibition. Infect. Immun. 16:471-475, 1977.
- 3. Zollinger, W. D., Mandrell, R. E. Outer membrane protein and lipopolysaccharide serotyping of <u>Neisseria meningitidis</u> by inhibition of a solid phase radioimmunoassay. Infect. Immun. <u>18</u>. In press, 1977.
- 4. Griffiss, J. M., Artenstein, M. S. The ecology of the genus Neisseria. Mt. Sinai J. of Med. 43:746-761, 1976.
- 5. Griffiss, J. M., Broud, D. D., Silver, C. A., Artenstein, M. S. Immunoepidemiology of meningococcal disease in military recruits. I. A model for serogroup independency of epidemic potential as determined by serotyping. J. Infect. Dis. <u>136</u>:176-186, 1977.

Comparative Animal and Human Responses to Meningococcal Vaccines Table 1.

Human Response	Immunogenic	YES	ON	NO	NO	YES & NO <sup>a</sup>
Hume	Safe	YES	YES	YES	YES	YES
Animal Response	Immunogenic	ON	ON	ON	YES	YES
Ani	Safe	YES	YES	YES	YES	YES
Vaccine	Description	A & C Capsular Polysaccharide	B Capsular Polysaccharide	E. coli Kl Capsular Polysaccharide <sup>a</sup>	Group B Type 2 Protein Extracts	Type 2 Outer Membrane Protein Plus C Polysaccharide

 $^{\mathrm{a}}$ Increases in bactericidal antibodies to group B meningococci were obtained, but these antibodies were not directed against the protein antigen.

Table 2. Inhibition of human serotype P2-specific bactericidal antibodies by OMC and its components following various treatments

MIC <sub>50*</sub>	> 500 > 500	3.3 >800 5.9	12 78 74 210**
Inhibiting antigen and treatment	99M purified LPS 99M group B polysaccharide	99M OMC Untreated Digested with pronase Precipitated with 80% ethanol	99M OMC dissolved in 3% DOC pH 8.8 then chromatographed on Sephadex G-100 in 1% DOC pH 8.8 All fractions pooled Protein peak Protein peak + LPS peak LPS peak (contained about 20% protein)

\*Minimal concentration that inhibited  $50\%-\mu g$  protein/ml \*\* $\mu g$  LPS/ml

Table 3. Properties of two new meningococcal group B polysaccharide-type 2 protein vaccines

	-	
Property	Vaccine Lot WZ-2 PA-	. Lot PA-3
Ratio Protein:polysaccharide	1:1	2:3
Percentage Nucleic Acid	9.0	1.3
Pyrogenicity in rabbits (largest non-pyrogenic dose-µg/kg)	1.0-2.0	0.37-0.5
Passed standard safety and toxicity tests in mice and guinea pigs	Yes	Yes

Table 4. Antibody Response of Rabbits to Two New Group B Polysaccharide-Type 2 Protein Vaccines.

		Vacci	Vaccine lot
Assay	Units*	WZ-2	PA-3
Bactericidal test vs. vaccine strain	reciprocal titer	22-1159**	22-1169
Farr test v.s. group B polysaccharide	ng antigen bound	40-100	58-122
Solid-Phase RIA v.s. vaccine strain OMC vaccine strain LPS	<pre>ug antibody/ml ug antibody/ml</pre>	1.4-253	1.5-518

\*Geometric mean of 7 rabbits

\*\*Prevaccination ~ 5 weeks post vaccination. Injections of 50 µg protein were given iv or sc at

0 and 3 weeks.

Table 5. Inhibition of Human Bactericidal Antibodies by Vaccine Lots WZ-2 and PA-3 $^{\rm a}$ 

Specificity of bactericidal antibody	Inhibitor	MIC (μg/m1) <sup>b</sup>
Group B		
polysaccharide	Lot WZ-2	12
	Lot PA-3	12
	Lot 3XCA	25
	E. coli Kl PS	50
		Serum A Serum B
Type 2 OM protein	Lot WZ-2	250 250
-,,, ,	Lot PA-3	30 < 62
	LPS	N.I. <sup>c</sup> N.I.
	B polysaccharide	N.I. N.I.
	OMC	8 30

<sup>&</sup>lt;sup>a</sup>Killing of strain 99M (B:P2,3,6:L3,7) in the radioactive bactericidal test.

 $b_{\mbox{Minimum}}$  concentration required to produce 50% inhibition.

 $<sup>^{\</sup>text{C}}\text{No}$  inhibition observed at 100  $\mu\text{g/ml.}$ 

Table 6. Cases of Group A Meningococcal Disease in Seattle, WA and Portland, OR

City	Nr.	Dates	S-R*	AI <sup>+</sup>	Child	Alcoholic
Seattle, WA	12	6/75-3/77	12	5	1	10
Portland, OR	2	1/76-6/76	4	0	1	7

\*Skid-road associated

+American Indian

‡ Daily heavy consumption and/or frequent binges

Table 7. Characterization and Designation of Sera Assayed for Group A Meningococcal Capsular Polysaccharide Antibody

Submitted by	Designation	Population	City* Age+	Age+	Nr
1. Univ. of Wash.	Early Seattle IHBC ATC (ATC-HV) (ATC-DI) (ATC-UI) (ATC-Staff) Cases	Skid-road  Non alcoholic Amer. Indians Alcoholic Treatment Center Harborview Medical Center Indian Detox. Center - Acute Indian Detox. Center - Conv. Staff personnel One month convalescent	w w w w w w	ययययययय ४००	75 50 108 (52) (16) (24) (16)
II. CDC N=112	Skid-road control Non-S-R control Carriers Contacts Cases Misc	Skid-road Pre-employment physical exam (non-skilled workers) Known Group A carriers Case contacts Cases Skid-road non-random	S S S&P S&P S&P S&P	A A A A A A A A A A A A A A C A A & C	44 44 34 7 10 3
III. U.S. Army Recruits N=148	8	Entrance sera - Ft. Ord, CA January 1975			148

495

Total \*S - Seattle, WA; P - Portland, OR +A - Adult; C - Child

40

Recovery of Organisms Elaborating Meningococcal Group A Capsular Polysaccharide Table 8.

Organism	No./No. tested	%	Population	City <sup>2</sup>
N. meningitidis (Gp A)	2/44 6/54	4.5 11.1	Skid-road controls <sup>2</sup> ATCC-HV <sup>2</sup>	s s
Cross-reacting enteric flora	2/44 2/14 1/34	4.5 14.2 2.9	Skid-road controls Case contacts Non Skid-road controls	S S&P S

 $^{1}$ S = Seattle; P = Portland

 $^2\mathrm{Differ}$  only in degree of alcoholism; age, sex, ethnic distribution equivalent; ATC-HV contains only alcoholics

<sup>3</sup>B. pumilus: 1-Skid road control; Str. faecalis; 2-Skid-road control and case contact from Portland; FTA + cocci (faecal smear positive by direct staining with fluorescein conjugated anti-Asss Ab; organism not recovered); 2-Case contact from Seattle and non Skid-road control

- Figure 1. A. Antigenic activity of group B polysaccharide vs. hydrolysis time at pH 5 and 37°C in three serological assays. Radioactive antigen binding assay of Farr, expressed as percentage of antigen bound by a hyperimmune horse serum (X). Concentration of antigen required to inhibit 50% of the bactericidal activity of normal human serum, expressed as a percentage of the initial activity (0). Concentration of antigen required to inhibit 8 units of human group B specific hemagglutinating antibody, expressed as a percentage of the initial activity ( $\square$ ).
- B. Capacity of group B polysaccharide to form a precipitin line in agar double diffusion tests against hyperimmune horse serum after hydrolysis for differentperiods of time.
- C. Estimated molecular size (0) of group B polysaccharide as judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis after hydrolysis for different periods of time. Free sialic acid, as measured by the thiobarbituric acid assay as a function of hydrolysis time ().

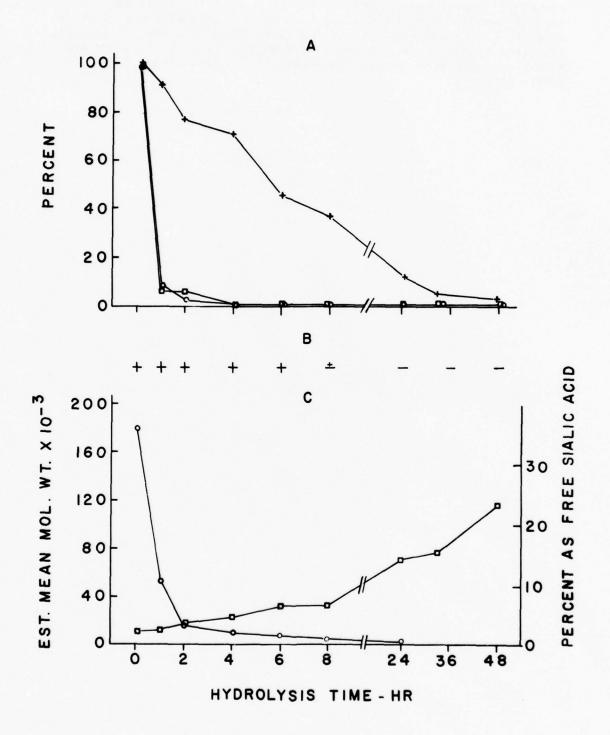
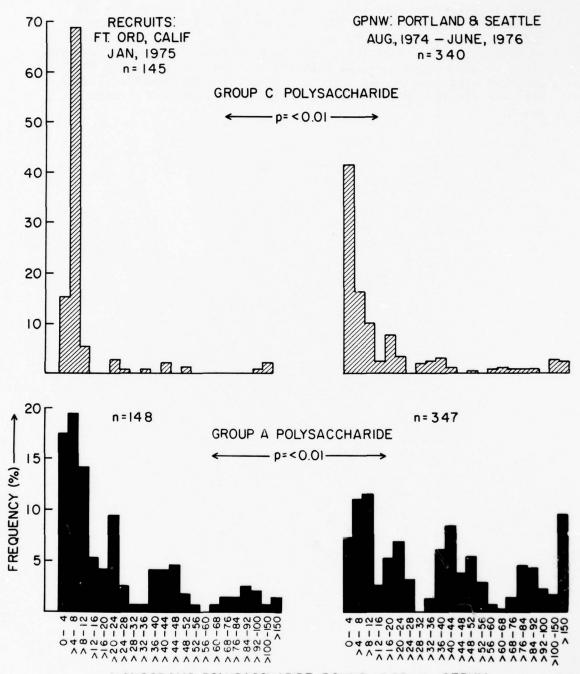
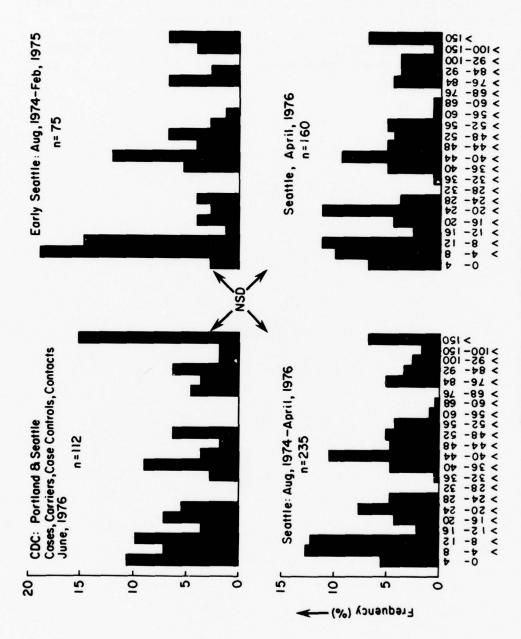


Figure 2. Prevalence of antibody to the group C (hatched) and group A (solid) meningococcal capsular polysaccharides among military recruits (left) and residents of Portland, OR and Seattle, WA (right), expressed as ngm of antigen bound by 0.05 ml serum. Differences are significant for both antigens (p=<0.01; Kolmogorov-Smirnov 2 - sample test).



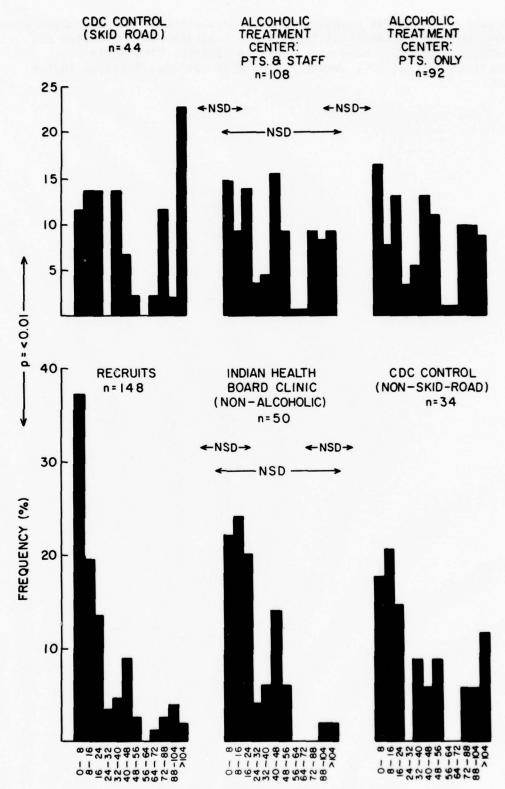
NANOGRAMS POLYSACCHARIDE BOUND / 0.05 mls SERUM

Figure 3. Prevalence of antibody to the group A meningococcal capsular polysaccharide (Asss) among populations surveyed by the Center for Disease Control (CDC) in portland and Seattle compared with that among populations in Seattle surveyed by the University of Washington (Seattle, left) and among Skid Road residents surveyed prior to the outbreak by the University of Washington (early Seattle) compared with populations surveyed in April, 1976 (Seattle, right). NSD: not sifnificantly different (Kolmogorov-Smirnov 2 - sample test).



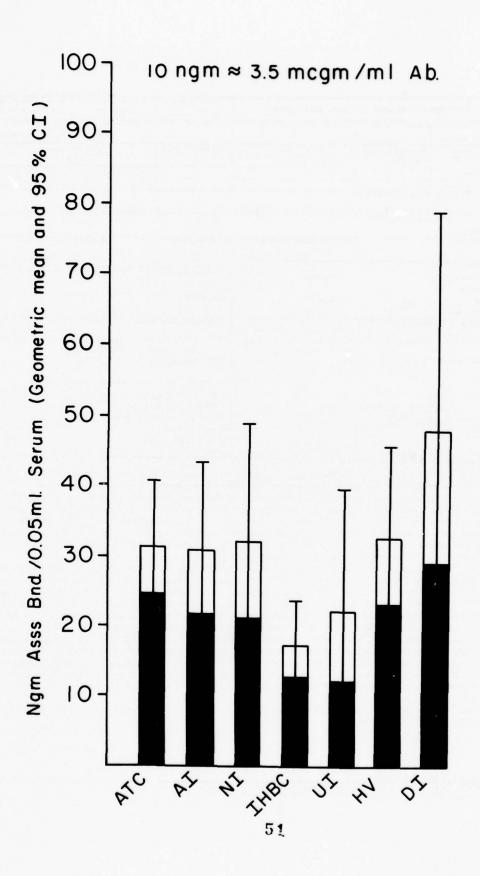
Nanograms Asss Bound / 0.05 ml Serum

Figure 4. Prevalence of antibody to group A capsular polysaccharide (Asss) among three non Skid Road populations (recruits, Indian Health Board Clinic and CDC non Skid Road control, bottom) and three Skid Road populations (CDC Skid Road Control, Alcoholic Treatment Center with and without treatment staff, top). Differences were not significant (NSD) among each group but were significant between groups (p=<0.01, Kolmogorov-Smirnov 2 - sample test).



Ngm Asss BOUND/O.05ml SERUM

Figure 5. Antibody levels in survey populations. ATC=alcoholic treatment centers; AI=American Indians ATC patients; NI=Non Indian ATC patients; IHBC=Non alcoholic American Indians; UI=Convalescent American Indians ATC; HV=Harborview ATC: DI=Acute American Indian ATC.



RESEARCH	AND TECHNOLOG	Y WORK UNIT	SUMMARY		OB 6523	77 09	30	DD-DF	CONTROL SYMBORAE (AR)636
DATE PREV SUMPRY	4. KIND OF SUMMARY	S. SUMMARY SCTY	S. WORK SECURITY	7. REGR		O'N INSTR'N	BE SPECIFIC D	CCESS !	LEVEL OF SU
76 10 01	H. Terminati	on U	U		NA	NI.	Kkyes [	) MO	A WORK UHIT
NO./CODES:*	PROGRAM ELEMENT		NUMBER	-	REA NUMBER		WORK UNIT	NUMBER	
CONTRIBUTING	61101A	3A161101A	191C	+	00		194		
CONTRIBUTING	<del>                                     </del>	<del> </del>		+					
	Security Classification Code	,,•							
(U) Develo	pment of an C	rgan Cultu	re Method	From	Intestina	1 Biops	ies		
SCIENTIFIC AND TE	CHNOLOGICAL AREAS								
002609		14. ESTINATED COM	PLETION DATE	TIS FUNI	HIG AGENCY		16. PERFORMA	HCE MET	HGD
76 01			09	l	1	1			
CONTRACT/GRANT			09	DA 10. RES	OURCES ESTIMATE	A PROFESS	IONAL MAN YRS	- hous	OS (In thousands)
DATES/EFFECTIVE:		EXPIRATION:			PRECEDING				
NUMBER:*				FISCAL	76	1_1	.5	_	72
C TYPE:	NA	d AMOUNT:		YEAR					
E KIND OF AWARD:	000441747104	f. CUM. AMT		m 050	77	1	.5		15
				-					
AME: Walter	Reed Army Ins	stitute of	Pathology	NAME:	Walter R			ute o	f Resear
ooness: Washi	ngton, DC 200	112		ADDRES	Washing	n of Par	20012		
washi	ington, be zee	.12			mashing	con, be	20012		
				PRINCIP	AL INVESTIGATOR	(Furnish SSAN	If U.S. Academic I	rne li fu tien;	
RESPONSIBLE INDIVID	UAL			HAME:	IAKEU	CHI, AK	IO, M.D.		
	MUND, GARRISC	ON, COL		TELEF	PHONE: 202-5	76-2024			
TELEPHONE: 20	2-576-3551			4	TE INVESTIGATOR				
				NAME:	ISSAC,				
Foreign int	elligence not	considere	ed	NAME:	,				
	BACH with Society Classif								
(U) Organ	Culture; (U)	Intestine;	(U) Phase	Micr	oscopy; (	U) Elect	tron Mic	rosco	ру
	elop a reliab								
large intes	tines of expe	rimental a	nimals may	be c	ultured a	nd main	tained w	ithou	t produc
	ions of funct								
studies on	interactions	between th	e mucosa a	nd va	rious bac	teria,	viruses	and m	icrobe-
derived tox	ins. These r	esults wil	l provide	new i	nformatio	n which	should d	clari	fy the
	s of acute di								
	entional morp								
as interfer	and histoche ence microsco	my and cin	ematograph	racer	methods	are bell	ig usea.	Met	nods suc
25 (U) 76-1	0-77 09 In o	rgan-cultu	red outs	our n	hase cont	rast and	as the v	on mi	croscone
observation	s have demons	trated tha	t shigella	toxi	n induces	cytopat	thic char	nges	in host
cells withi	n 30 minutes	after toxi	n administ	ratio	n. An ul	trastru	ctural le	evel	of in-
testinal ep	ithelial cell	s showed s	hortened b	lunt	microvill	i and su	vollen cy	ytop1	asm con-
taining dec	reasing number	rs of ribo	somes, swe	lling	of both	rough ar	nd smooth	n end	oplasmic
reticulum a	nd moderately	altered m	itochondri	a wit	h opacifi	ed matr	ix and of	scur	e intra-
mitochondri	al granules.	hosis of	nges stron	gly s	uggest th	at shige	ella toxi	in is	capable
or arcering	protein synt	nesis of c	urtured in	testi	nai epith	eriar ce	ells.		
For technic	al reports, s	ee Walter	Reed Army	Insti	tute of R	esearch	Annual I	roar	ess
Report, 1 J	ul 76-30 Sep	77.			or K	- Jear off		rogi	
			52						
			26						

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 194 Development of an Organ Culture Method From Intestinal Biopsies

Investigators

Principal: Akio Takeuchi, M.D. Associate: Gertrude Issac

## Description

To develop a reliable and reproducible method for the organ culture of the small and large intestine of experimental animals and man. The cultured gut will be employed in: (1) Studies by various parameters of responses of the gut mucosa and submucosa to various enteric microbes and microbe-derived toxins; special attention will be paid to cinematographic recordings of as well as conventional static observations on various cellular interaction of the gut epithelium mucosa with various microbes and toxins. (2) Studies of replication sites of certain enteroviruses in the cultured gut.

These studies should provide valuable new information which will clarify as yet unsolved problems in pathogenesis of acute infectious diarrheal diseases common in military personnel at home and overseas.

#### Background

Organ culture techniques have proven to be of considerable value in the study of respiratory tract infections in man and animals. Recently, interest has developed in the organ culture of gut tissues for the cultivation of enteric viruses. Rubenstein and Tyrrell (1971), Dolin and Stenhouse (1970), and Derbyshire and Collins (1971) provided evidence of the multiplication of viruses in organ cultures of the small intestine of the human embryo. Dolin, et al. (1972) reported that viral antigens from viruses which belong to different viral groups were successfully detected in human fetal intestinal organ cultures by immunofluorescent techniques. Kagnoff, et al. (1972), in their organ culture study of adult rabbits, have reported that the metabolic function and synthesis of macromolecular substances including secretory IgA were still active after 24 hours culture in a Petri dish. Finally, Eastwood and Trier were able to culture the human small intestine of normal subjects and patients with ulcerative colitis up to 24 hours (Eastwood and Trier, 1973).

Most of the chambers used by the above investigators appeared adequate for the growth of certain viruses and fro evaluation of limited metabolic activities in the cultured bowel. These chambers do not allow for an immediate and accurate and also sequential method of morphologic evaluation of intestines growing in cultures. This shortcoming prompted the development of a new chamber allowing a convenient observation during actual organ culture. The chamber we have developed by modifying Rose's chamber provides easier handling of tissues and better visualization of growing cells and tissues under the phase contrast microscope than conventional culture chambers. The viewing unit consists of a phase microscope, which can be attached to a time-lapse cinematographic instrument, enclosed in a plastic housing connected with a thermo-control device which maintains the temperature of the unit at 37°C (Takeuchi, et al., 1974).

## Progress

Utilizing our new culture chamber together with the conventional Falcon cahmber, we have continuously cultured fetal small and large intestine. By our method, the fetal gut can grow well without it for up to 48 hours and shows no structural alteration. We have obtained an excellent growth and survival of intestinal epithelial cells, the most sensitive cell population of the gut, in the culture medium 199 with 20-10% adult horse serum containing either gentamicin or the combination of streptomycin and penicillin in 100%  $CO_2$  at  $37^{\circ}C$ .

Effects of Crude Shigella dysenteriae Toxin on Cultured Gut

Utilizing the above cultured technique, we were able to observe changes of epithelial cells of cultured small intestine at ultrastructural level. 1 ng of crude toxin was capable of producing cytoplasmic alterations within 30 minutes (Fig. 1).

They consisted of blunting, blistering and ballooning of microvilli (Fig. 2), swelling and opacifying mitochondria with deranged cristae, swelling and dilatation of both smooth and rough endoplasmic reticulum with decreasing numbers of ribosomes (Fig. 3). These EM findings suggested : 1) markedly altered absorption of enterocytes; 2) abnormal mitochondrial function - oxidative phosphorization; 3) altered protein synthesis.

Further studies were needed to clarfly the precise nature of these interesting changes with biochemical and ultrastructural method.

Due to the shortage of research personnel this project had to be terminated.

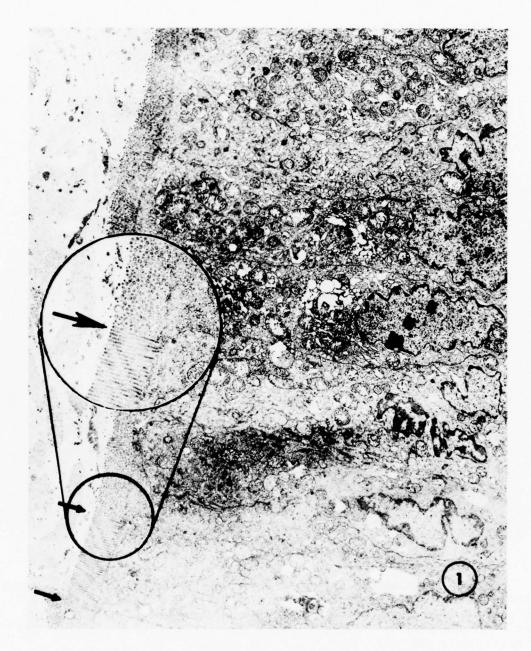


Fig. 1. Electron micrograph of mid-villus epithelium of cultured jejunum. Epithelial cells are diffusely involved by a variety of cytoplasmic alterations. Blistering and ballooning of microvilli are obvious in most of the epithelial cells except for a cell whose microvilli remain unaltered (sandwiched by small arrows). Changes in underlying cytoplasm include swelling of mitochondria, and dilated cisternae of ER and vesicles of Golgi (See Figs. 2 and 3); X3,200. Circled inset illustrates higher magnification of an intercellular junctional area (large arrow) showing unaltered microvilli at one cell and ballooning microvilli at another. X6,500.

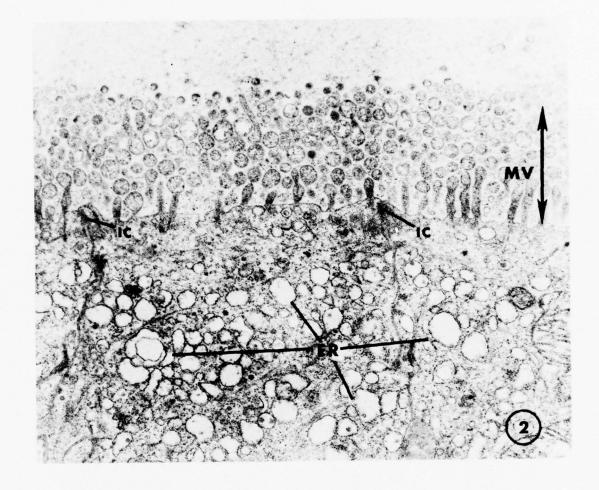


Fig. 2. High magnification of the brush border of enterocytes under the effects of shigella toxins. The microvilli (MV) show a variety of changes including ballooning, blistering and fragmentations. Endoplasmic reticulum (ER) is dilated, however, the intercellular junction (IC) remains unaltered. X18,000.

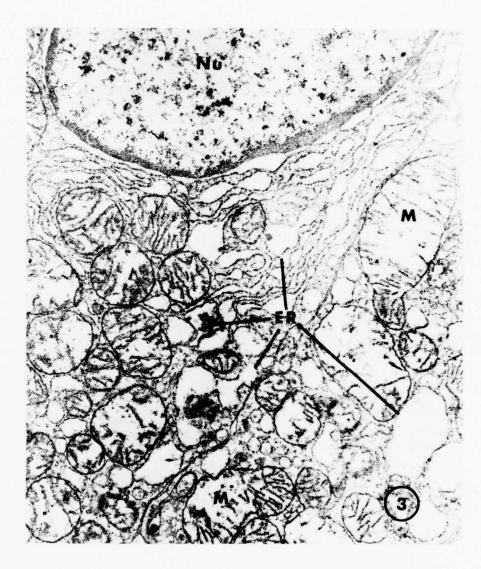


Fig. 3. Altered mitochondria in a jejunal epithelial cell at mid-villus area. Both rough and smooth endoplasmic reticulum (ER) are dilated with decreased numbers of ribosomes. Mitochondria (M) are swollen and the matrix is opaque. The cristae are altered. Nu=nucleus. X18,000.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 194 Development of an Organ Culture Method From Intestinal Biopsies

## Literature Cited

#### References:

- 1. Derbyshire, J.B. and Collins, A.P. The multiplication of Talfan virus pig intestinal organ cultures. Res. Vet. Sci., 12: 367, 1971.
- 2. Dolin, R., et al. Establishment of human fetal intestinal organ culture for growth of viruses. J. Inf. Dis., 122: 227, 1970.
- 3. Rubenstein, D., Milm, R.G., Buckland, R. and Tyrrell, D.A.J. The growth of the virus of epidemic diarrhea of infant mice (EDIM) in organ culture of intestinal epithelium. Br. J. Exp. Path., 52: 422, 1971.
- Kagnoff, M.T., et al. Organ culture of rabbit small intestine: Prolonged in vitro steady state protein synthesis and secretion and secretory IgA secretion. Gastroenterol., 63: 541, 1972.
- Eastwood, G.L. and Trier, J.S. Epithelial cell renewal in cultured rectal biopsies in ulcerative colitis. Gastroenterol., 64: 383-390, 1973.
- 6. Eastwood, G.L. and Trier, J.S. Organ culture of human rectal mucosa. Gastroenterol., 64: 375-382, 1973.
- 7. Takeuchi, A., Asaka, T. and Takeda, T. Cinematographic observations on the bowel in organ culture. Fed. Proc., 33: 328, Abs., 1974.

RESEARCH	AND TECHNOLOG	WORK UNIT S	UMMARY			77 09		The state of the s	CONTROL SYMBOL
3. DATE PREV SUM'RY	4. KIND OF SUMMARY	S. SUMMARY SCTY	. WORK SECURITY	7. REGR	DB 6539	DISB'N INSTR'N		DATA-	S. LEVEL OF SUM
76 10 01	K. Completion	PROJECT	U		NA	NL	☑ YES	□ NO	A WORK UNIT
A PRIMARY	61101A	3A161101A9		TASK	00	-		96	<u>'</u>
b. CONTRIBUTING	OTTOTAL	SATIOTIONA.	,,,,,		00				
c. CONTRIBUTING									
	security classification code  emical Charac  CHHOLOGICAL AREAS*		of Arbovir	us Ar	ntigens				
002300 Bio	chemistry 0	02600 Viro	OGY 0101	00 M	icrobiol	ogy	16. PERFOR	MANCE MET	ноп
74 07		77 09		D/	<u> </u>	1	C. In-	-House	
& DATES/EFFECTIVE:	NA	EXPIRATION:		18. RES	PRECEDING	TE & PROFE	SSIONAL MAN Y	RS & FUN	IDS (In thousands)
b. NUMBER:*				FISCAL	76		1.0		111
G TYPE:		& AMOUNT:		YEAR	CURRENT				
e. KIND OF AWARD:		f. CUM. AMT.			77		1.0		131
MAME: Walter Reed Army Institute of Research Walter Reed Army Institute of Resear									
Div of CD&I									f Research
Div of CD&I  ADDRESS:* Washington, D.C. 20012									
PRINCIPAL INVESTIGATOR (Furnish SEAN II U.S., Academic Institution)									
RESPONSIBLE INDIVIDUAL NAME: BANCROFT, LTC William H.									
MAME: RAPMUND, COL Garrison TELEPHONE: (202) 576-3757									
21. GENERAL USE ASSOCIATE INVESTIGATORS									
Foreign intelligence not considered.									
TECHNICAL OBJECT	TIVE, 24 APPROACH, 25.	PROGRESS (Fumial in	IIIIIIUNO I OQY :	entitled by	number. Precede	atnology	A Security Classifi	ication Code	.)
23 (U) To (	define the an	tigenicity	and immuno	genio	city of	the stru	ictural a	and no	nstruc-
	ins of arbovi the immune re								
tions.	the finingite is	esponse, pr	oucceron a	nu n	munopa	norogy c	)	11 43 1	
24 (U) Ant	tigens from v	irions or i	nfected ce	11s a	are sepa	rated ar	nd purif	ied by	ultra-
centrifugati	ion, column cl	romatograp	hy, and is	oeled	tricfoc	using.	Sensitiv	ve ass	ays for
	f viral antig								
	tisera to them and modificat			ems	are inve	stigated	i for eva	iluati	ng
	10 - 77 09 1			ositi	ive resu	lts incl	ludes the	rese	arch and
	of (1) method								
antigenic co	omponents with	nout extens	ive denatu	ratio	on, (2)	purifica	ation and	i sepa	ration of
	ntigens, (3) s pecificity and								
	virulence, pro								
virus vaccin			nclude our						
	res developed								
for arboviru	us infections	of militar	y importan	ce.	These i	nvestiga	itions an	re to	be
	d into the ma ended to incl								
	as they are								
technical r	eport, see Wa	lter Reed	Army Instit	tute	of Resea	arch Ann	ual Prog	ress F	Report,
1 Jul 76 -	30 Sep 77.				59				
					0.0				
DD FORM 149	PREVIOUS		IS FORM ARE OF	SOLF	E. DD FOR	MS 1498A. 1	NOV 88		
DUI MAR 66 145	AND 1498-1	, 1 MAR 68 (FOR	ARMY USE! ARE	OBSOL	ETE				

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH Work Unit 196, Biochemical characterization of arbovirus antigens

## Investigators:

Principal: LTC W.H. Bancroft, MC; J.M. Dalrymple, Ph.D.

Associate: COL P.K. Russell, MC; W.E. Brandt, Ph.D;

SP5 S.A. Harrison; SP4 S.M. Crump; B.H. Mann;

G.P. Onley; R.J. Jackson

# Description

To define the antigenicity of arbovirus structural and nonstructural components as potential immunogens of prophylactic value. To isolate and evaluate virus components as antigens for evaluation of immune status and description of the role of such antigens in the immune response and immunopathology of arbovirus infections. Studies have emphasized the biochemical and biophysical characterization of arbovirus antigens and the development of appropriate model systems for evaluating protection and the immune response.

### Progress

I. Investigation of Parameters Affecting Flavi, virus Propagation and Replication to High Titers.

A major problem relevant to obtaining flavivirus antigens in quantities sufficient to perform antigenic analysis is the low virus yield obtained from infected cultures. Numerous experiments were designed to investigate the basic parameters affecting virus yield; however, no results were obtained that would allow a significant increase in virus titer or yield. Japanese encephalitis virus (JEV) and dengue-2 virus were examined in a variety of cell lines including LLC-MK2 and VERO (continuous monkey kidney cells) BHK 21/15 (baby hamster kidney) and primary chick embryo cell (CEC) fibroblasts.

JEV was not released from infected cells into the supernatant medium until 10-14 hours post infection and maximum titers (approx.  $10^7$  PFU/ml) were obtained within 22-26 hours. Infection of LLC-MK2, BHK & CEC yielded essentially identical results; however, Vero cells did not release virus until 22 hours post infection and did not achieve maximum titers until 36-50 hours. Results with dengue-2 virus were similar with earliest virus production at 14-18 hours, maximum titer (approx.  $10^6$  PFU/ml) at 26-30 hours, a slight delay in virus replication by Vero cells and the notable exception that dengue-2 virus did not replicate in CEC cultures.

In contrast to the growth curve studies utilizing infected suckling mouse brain suspensions as infective inoculum, virus passaged in each of the cell lines (LLC-MK2, BHK, Vero) using 24 or 48-hour harvest of infected supernatants as infective inoculum for new cultures of the same cell type failed to significantly increase virus yield. Passage through six consecutive cell-to-cell transfers actually resulted in reduced virus yields even though slight titer elevation at passage 2-3 suggested some adaptation. Decreased titers at passage six did not appear due to an enrichment of defective interfering virus particles since pre-infection of cells with these passaged virus suspensions did not reduce the normal yield when these same cells were subsequently infected with mouse brain-propagated suspension. Reduced virus yields upon cell-to-cell passage were most probably due to the diminishing multiplicity of infection (MOI) that occurred upon passage. Experiments specifically designed to investigate the effects of MOI clearly showed that maximum virus yields were obtained at an MOI of one. Yields were slightly decreased at MOI's of ten or greater and significantly reduced at MOI's of 0.1 or less. All of the results discussed were consistent for each of the cell lines employed and applied to JEV and dengue-2 virus strains to include New Guinea "C" strain, PR 159-GMK6 (parent for dengue-2 vaccine) and the S-1 vaccine strain. Current conditions for flavivirus propagation appear optimal and our failure to significantly increase virus yields simply means that future efforts should be directed toward producing large quantities of virus for subsequent concentration and purification.

II. Development of Polyacrylamide Gel Electrophoresis (PAGE) Systems for Analysis of Structural and Non-Structural Arbovirus.

Research on the separation of arbovirus antigens is almost totally dependent upon a sensitive and reproducible PAGE system for resolving both structural and non-structural proteins of these viruses. Such systems have been developed and applied to a great many arboviruses currently under investigation in the Department of Virus Diseases (DVD). Specific details and procedures reflect the compilation and integration of numerous published reports by others and are (or will soon be) published in detail in publications from DVD; however, the interpretation of much of the data to follow requires some description of the methods and their application.

Two basic discontinuous PAGE systems have been adopted and are generally run as slabs. Both employ acrylamide: DATD (N, N'-Dially-tartardiamide) at a 30:1.3 ratio. DATD replaces the convention bisacrylamide and yields a more porous and flexible gel that must be used at higher concentration to achieve the same separations, but, in our lab, allows greater resolution and dries without cracking. Both gels are discontinuous, using a 0.375 M resolving gel pH 8.9, a 0.125 M spacer gel pH 6.7, and both contain urea and sodium lauryl sulfate (SLS). Alphavirus proteins are best resolved on a 14% resolving gel with a 3.6%

spacer; however, the flaviviruses and their intracellular non-structural proteins can only be resolved on a single gel using a gradient of 10-20% for a resolving gel with the same 3.6% stacking or spacer gel. Tris-HCl buffer is used throughout for pH control and the electrode buffer contains glycine and SLS in addition to the Tris-HCl buffer.

Proteins labelled with  $^{35}\mathrm{S}$  or  $^{125}\mathrm{I}$  can be visualized using classical methods of autoradiography. Tritium ( $^{3}\mathrm{H}$ ) or low level  $^{14}\mathrm{C}$  labelled proteins are best detected by fluorography. Basically, the method involves dehydration of the gels in two changes of DMSO followed by impregnation of the gel with the primary fluor PPO by soaking in a 20% PPO/DMSO (w/w) solution. The PPO is precipitated by immersing the gel in water and the gel is subsequently dried on filter paper. Dried gels are placed on Kodak X-Omat R film (XR-2) and placed at -70°C during the exposure period. Single bands containing greater than 2000 cpm can be visualized on the developed film following 24-48 hours exposure at -70°C. Standard marker proteins used to standardize the gel systems included cytochrome C(12,5000 mw), chymotripsinogen A (25,000 mw), hen albumin (45,000 mw), aldose (158, 000 mw) and catalase (240,000 mw). Migration of these standard proteins in a slab, 10-20% gradient PAGE gel of 15.5 cm length yielded RF values of 0.85, 0.65, 0.55, 0.51, and 0.42 respectively. Radiolabelled vesicular stomatitis virus (VSV) was frequently employed as an internal marker for these gel runs with the four proteins of this virus controlling abnormal electrophoresis patterns, gel abnormalities etc.

The adoption of these discontinuous slab PAGE systems with associated fluorography and autoradiography have greatly simplified out viral protein analysis requirements.

III. Solubilization of JEV Infected CEC Antigens and Animal Immunization.

Flavivirus infected cells contain numerous non-structural viral proteins which are virus-coded and presumably antigenic yet are never incorporated into virions. A major problem in the investigation of these "antigens" is the inability to selectively remove the proteins from the infected cells for antigenic characterization. A variety of extraction procedures were therefore applied to JEV infected CEC cultures in an attempt to solubilize these proteins for further study.

Infected cells were radiolabelled with  $^3\text{H-amino}$  acids and the distribution of these intracellular proteins was monitored during extraction using gradient discontinuous slab PAGE. Extraction procedures included combinations of a variety of salt concentrations, both ionic and non-ionic detergents, reducing agents such as 2-mercaptoethanol (2ME) urea and chloroform-methanol mixtures. Most successful among the solubilization procedures was treatment with 0.5-2.0% SLS in combination with 0.1-2.0% 2ME. These extractions released 80-100% of the radioactive counts from infected cell cultures. Other procedures which resulted in

the release of 40% or more of the protein radioactivity included 8 M urea, 0.3 M lithium 3,5 diiodosalicylate, and various combinations of these reagents with 2ME.

Discontinuous gradient PAGE slabs revealed a complete spectrum of structural and non-structural JEV proteins in SLS-2ME solubilized material. Similar preparative slabs containing these proteins were sliced into 10 segments representing the various proteins and were used to immunize rabbits. Gel suspensions prepared with the aid of a tissuemizer were mixed with Freuud's complete adjuvant and infected intradermally at 4 sites. Rabbits were bled 10 days following a three-week booster injection and examined for anti-JEV antibody activity. No neutralizing or hemagglutination-inhibiting antibody activity was detected in any of these sera. Complement-fixation tests did reveal antibody in sera from animals immunized with gel slices containing the major envelope glycoprotein of the virion and those immunized with the non-structural protein designated NV-3. Trace antibody was observed in other sera.

These data would suggest that the immunogenic potential of such fractions is not great, possibly due to denaturation of these proteins during the extraction procedures. Examination of these sera using more sensitive radio-immune assay procedures and a variety of antigen sources is contemplated.

IV. Comparative Intracellular Protein Profiles of Flavivirus Infected Cells Using Gradient Discontinuous PAGE Slabs.

The intracellular proteins of those flaviviruses which have been examined have been described using chemical inhibitors of normal host cell protein synthesis such as actinomycin-D etc. Since most such inhibitors have been shown to have a deleterious effect on infectious virus yield, it becomes important to examine the intracellular profile of virus specified proteins in uninhibited cells if possible. The development of slab discontinuous gradient PAGE systems allowed the examination of many samples simultaneously and prompted a detailed study of virus specified protein synthesis in uninhibited cells over the time course of infection. Radioactive amino acid precursors in a pulse-chase experimental design were used to examine the kinetics of both JEV and dengue-2 virus protein synthesis relative to those of mock infected normal host cells.

During the period of exponential virus release, amino acid pulses as short as 3 minutes were incorporated into virus proteins; however, 30 minutes was used as a routine pulse for visualizing all of the non-structural proteins. Background levels of normal host cell protein synthesis precluded the use of this method for viral protein examination earlier than 12 hours post infection, although the major virion envelope glycoprotein (V-3) and the larger non-structural proteins (NV-5, NV-4) could be detected. From 12-20 hours post infection, a definite decrease in normal host cell protein synthesis could be

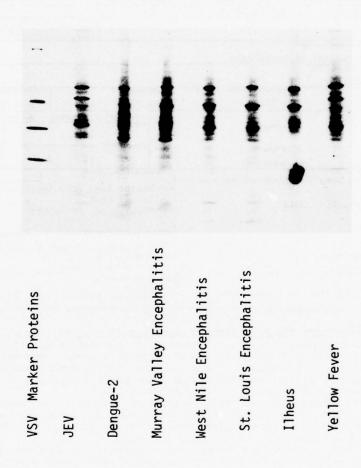
observed in virus infected cells. Although this decrease was not equal among all of the host cell proteins, virus specified proteins could be easily distinguished. Cultures pulsed at 12-16 hours post infection revealed a clear pattern of virus specified proteins. Infected cells examined at the intervals through 48 hours post infection failed to reveal any significant change in the virus specified protein profile, although decreased host cell protein synthesis yielded cleaner profiles at the later times.

Pulse chase experiments failed to demonstrate clear evidence of post-translational cleavage as a functional means of virus specified protein synthesis; however, many of these viral proteins exhibited differential rates of synthesis throughout the replication cycle. Most notable among the differences observed was the disappearance of the major envelope glycoprotein V-3 with extended chase periods. This observation is assumed to reflect assembly and release of complete virions into the supernatant fluids. Both JEV and dengue-2 virus infected cells appeared similar with respect to the kinetics of virus protein synthesis, release, etc. The development of procedures and background data on the kinetics of flavivirus protein synthesis provides a firm laboratory base for examination of the nature of temperature-sensitive dengue vaccine virus replication defects, antigenic and/or strain variation among flavivirus strains and the relationship of protein synthesis to intracellular antigen development.

Throughout the course of these studies, it became quite apparent that the virus specified intracellular protein profiles of JEV and dengue-2 virus infected cells were not identical even though the kinetics of protein synthesis appeared quite similar. Other flaviviruses were selected and examined by the discontinuous gradient slab PAGE in an attempt to detect differences in the mobility of the virus specified intracellular proteins. Most flaviviruses were thought to be similar if not identical with respect to molecular weight estimations of the non-structural virus proteins.

An autoradiograph comparing seven different flavivirus infected BHK 21/15 cultures is shown in Figure 1. Vesicular stomatitis virion proteins are included for reference. Although certain host cell proteins are still present in these preparations taken 20 hours post infection, differences in the major virus proteins can be detected. The largest non-structural protein, NV-5, migrates approximately the same in each of the virus preparations; however, the NV-4 protein of JEV and yellow fever virus is considerably larger than the others. Without specifically delineating each of the differences, it is apparent that this technique allows differentiation of many of these flaviviruses based on differences in the relative mobilities of their intracellular proteins. Recalling that these preparations were examined 20 hours post infection, it is conceivable that gradient slab discontinuous PAGE and fluorography can be expanded to yield a flavivirus rapid identification test.

FIGURE 1. - Gradient Slab Discontinuous PAGE of BHK21 Cells Infected with Representative Flaviviruses.



TATE PROPERTY IN PROPERTY OF A SUMMAY EAST OF A DOOR SCENTY OF A SUMMAY CONTROLLED TO SERVICE THE PROPERTY OF A SUMMAY	RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY					C 6430	77 09 30		DD-D	CONTROL SYMBO R&E(AR)636	
***Control of the product is a process of the profession of the product is a process of the profession of the profession of the product is a process of the profession of the profession of the profession of	Account to the second second				7. REGRA	DING. B. D		CONTRACTOR	ACCESS	A WORK UNIT	
Continuoring   Cont	NO./CODES:*	PROGRAM ELEMENT	PROJEC	T NUMBER	TASK A			CAVE2		A	
CONTINUE CON							197				
(U) Molecular Biology of Rickettsia  DIOJOD Microbiology  TATFOYTE  TO 9  DA   C. In-House  C. In-House  Contract/Conner  TO 07  TO 9  DA   RESPONSE ESTIMATE   PROFESSIONAL MAN IN PROCESSIONAL MAN IN PROCES	CONTRIBUTING				1						
(U) Molecular Biology of Rickettsia    Continue was recommonded. Americal   Continue	CONTRIBUTING										
DIOLOGO Microbiology  TRATIGUEST TO THE CONTROL OF THE STREET TO THE STREET CONTROL OF T	TITLE (Procede with	Security Classification Code	,•								
ONTERIOR PROPERTY OF THE ALTERNATION OF THE ALTERNA			f Ricketts	ia							
TREAD TO THE CONTRACTION OF THE EXPENSATION OF THE PROPERTY OF											
TOTAL	010100 Mic:	robiology	Tia Frymaren con	10: EVIOU BAVE	Tie sumo	NC ACENCY		Tre menenne	W W.		
Under the properties of sub-cellular components responsible for susce itity to disease in laboratory animals. Studies are aimed at improving rickettsial accines to prevent disease in troops operating in the field.  4. (U) 1. Mutagenization and selection of appropriate rickettsial strains by tisst ultrure techniques. 2. Growth and purification of rickettsiae followed by polyacryl ide gel electrophoresis of sub-cellular fractions, 3. Use of genetically homogened not considered to produce detectable mutants. The tissue culture techniques and molecular weight determinations conducted. 3. Over 30 inbred strains with contents of inchestical services and beauty of inchesion and purification of rickettsiae in the field.  5. (U) 76 10-77 09 1. Conditions for selection of temperature sensitive mutants of ickettsiae were established, but exhaustive mutagenesis with chemical agents failure or produce detectable mutants. The tissue culture technology developed, however, if ending in the fine produce detectable mutants. The tissue culture technology developed, however, if ending in the fine produce detectable mutants. The tissue culture technology developed, however, if ending serviced and molecular weight determinations conducted. 3. Over 30 inbred strains in the followed proprise focused on isolation and identified on produce detectable mutants. The tissue culture technology developed, however, if ending in the field and produce detectable mutants. The tissue culture technology developed, however, if ending send for antigenic analysis of naturally occurring variants of rickettsiae. 2 inchemical studies of rickettsial macromolecules focused on isolation and identified on produce detectable mutants. The tissue culture technology developed, however, if ending and molecular weight determinations conducted. 3. Over 30 inbred strains in the feed and molecular weight determinations conducted. 3. Over 30 inbred strains in the feed and molecular weight determinations conducted. 3. Over 30 inbred strains in the feed and molecular we				arce flow on te			,				
**************************************			17 09		-		<del></del>		1		
REPORTING TO CONTROL T		NA	5×818×7104		16. RESC		E & PROFESS	SIONAL MAN YRS	h Fu	105 (In thousands)	
TELEPHONE CONCESS.  Washington, DC 20012  ***MANNET**  ***Washington, DC 20012  ***MANNET**  ***Washington, DC 20012  ***MANNET**  ***M		NA	EATINATION:			76		2		107	
***Memory awano**  ***Walter Reed Army Institute of Research  ***Walter Reed Army Institute of Research  ***Walter Reed Army Institute of Research  ***PREVIOUS MARKET REMAINS ARE ARE THE TREE ARE ARE THE LANGUAGE RESEARCH TO THE TREE ARE THE TREE ARE ARE THE LANGUAGE RESEARCH TO THE TREE ARE THE TREE AR	CONTRACTOR OF THE PARTY OF THE		4 AMOUNT:			CURRENT	+	+			
Walter Reed Army Institute of Research  Walter Reed Army Institute of Research  Mante.  Walter Reed Army Institute of Research  Div of CD&I  ***Mark Walter Reed Army Institute of Research  Div of CD&I  ***Mark Walter Reed Army Institute of Research  Div of CD&I  ***Mark Walter Reed Army Institute of Research  Div of CD&I  ***Mark Walter Reed Army Institute of Research  ***Mark Repinder Mark Walter Reed Army Institute of Research  ***Mark Repinder Mark Walter Reed Army Institute of Research  ***Mark Repinder Mark Walter Reed Army Institute of Research  ***Mark Repinder Mark Walter Reed Army Institute of Research  ***Mark Repinder Mark Walter Reed Army Institute of Research  ****Mark Repinder Mark Walter Reed Army Institute of Research  ****M						77					
Washington, DC 20012  ***Washington, DC 20012  ***Washington, DC 20012  ***Washington, DC 20012  ****Washington, DC 20012  ****Washington, DC 20012  ****Washington, DC 20012  *****Washington, DC 20012  *****Washington, DC 20012  ******Washington, DC 20012  *********************************		ORGANIZATION	1	<del></del>	20. PERF	ORMING ORGANI	ZATION				
Div of CD&I    Contest.   Washington, DC 20012     Div of CD&I	Walter	Reed Army Ins	stitute of	Research	- June 10	altar Do	ad Armir	Inatitut		Pagagara	
***Somewashington, DC 20012  ***Repromibilit Modification (Col., MC								Institut	.6 01	Research	
**RINCIPLE HOUSE TOOL ACT   Formula MANN   U.   Account   Invitation    **RECEPTIONE 202-576-3551  **GENERAL USE**  Foreign intelligence not considered.  **RELEPTIONS (Procede RACH STA Secont) Classification code)**  Relationship: (U) Structure—Antigenicity  **TECHNICAL OBJECTIVE** 24 APPROACH. 25 PROGRES (Promit individual program: individual procession) of rickettsiae to provide strains with optimum immu enicity. 2. Isolation and purification of sub-cellular components responsible for liciting immunological protection. 3. Determination of the genetic basis for susce ility to disease in laboratory animals. Studies are aimed at improving rickettsial accines to prevent disease in troops operating in the field.  4. (U) 1. Mutagenization and selection of appropriate rickettsial strains by tissulture techniques. 2. Growth and purification of rickettsiae followed by polyacrylide gel electrophoresis of sub-cellular fractions. 3. Use of genetically homogeneous more disease were established, but exhaustive mutagenesis with chemical agents failed oproduce detectable mutants. The tissue culture technology developed, however, it eing used for antigenic analysis of naturally occurring variants of rickettsiae.  5. (U) 76 10-77 09 1. Conditions for selection of temperature sensitive mutants of ickettsiae were established, but exhaustive mutagenesis with chemical agents failed oproduce detectable mutants. The tissue culture technology developed, however, it eing used for antigenic analysis of naturally occurring variants of rickettsiae. 2 iochemical studies of rickettsial macromolecules focused on isolation and identificated members are assistance was dominant and coded for by one or a closely linked user of autosomal genes. For technical report see Walter Reed Army Institute of the control of autosomal genes. For technical report see Walter Reed Army Institute of the control of autosomal genes. For technical report see Walter Reed Army Institute of the control of autosomal genes.	Washin	ngton, DC 200	012					20012			
HAME: Rapmund, Garrison, COL, MC  ***CLEMPONE*202-576-3551  **GENERAL USE**  Foreign intelligence not considered.**  **Freeign intelligence not considered.**  **CENTRONG**(Proceeds EACH with Secondin Clearlikenian Code)**  (U) Genetics; (U) Biochemistry; (U) Structure-Function Code)**  **Relationship: (U) Structure-Antigenicity**  **Relationship: As Amproach. is **Progress (Promiss for sub-cellular components responsible for liciting immunological protection. 3. Determination of the genetic basis for susce ility to disease in laboratory animals. Studies are aimed at improving rickettsial accines to prevent disease in troops operating in the field.  **4. (U) 1. Mutagenization and selection of appropriate rickettsial strains by tissulture techniques. 2. Growth and purifications. 3. Use of genetically homogeneous mired mice to determine basis of rickettsial virulence.  **5. (U) 76 10-77 09 1. Conditions for selection of temperature sensitive mutants of ickettsiae were established, but exhaustive mutagenesis with chemical agents failed opposite and molecular weight determinations conducted. 3. Over 30 inbred strains were ecognized and molecular weight determinations conducted. 3. Over 30 inbred strains were ecognized and molecular weight determinations conducted. 3. Over 30 inbred strains were ecognized and molecular was dominant and coded for by one or a closely linked luster of autosomal genes. For technical report see Walter Reed Army Institute of the control of the c					"	doningto	, 50 2	20012			
Foreign intelligence not considered.  Relationship: (U) Structure—Antigenicity.  Technical contents of the property of the provide strains with optimum immu enicity. 2. Isolation and purification of sub-cellular components responsible for liciting immunological protection. 3. Determination of the genetic basis for susceility to disease in laboratory animals. Studies are aimed at improving rickettsial accines to prevent disease in troops operating in the field.  4. (U) 1. Mutagenization and selection of appropriate rickettsial strains by tissulture techniques. 2. Growth and purification of rickettsiae followed by polyacrylide gel electrophoresis of sub-cellular fractions. 3. Use of genetically homogeneous detectable mutants. The tissue culture technology developed, however, it engused to produce detectable mutants. The tissue culture technology developed, however, it engused for antigenic analysis of naturally occurring variants of rickettsiae. 2 inchemical studies of rickettsial macromolecules focused on isolation and identification employing polyacrylamide gel electrophoresis. Over 17 individual proteins were ecognized and molecular weight determinations conducted. 3. Over 30 inbred strains in feeding indicated resistance was dominant and coded for by one or a closely linked luster of autosomal genes. For technical report see Walter Reed Army Institute of luster of autosomal genes.					PRINCIP	AL INVESTIGATO	R (Fumiah SSAN	II U.S. Atamale	[no! fution	•	
Foreign intelligence not considered.  Relationship: (U) Structure—Antigenicity.  Technical contents of the property of the provide strains with optimum immu enicity. 2. Isolation and purification of sub-cellular components responsible for liciting immunological protection. 3. Determination of the genetic basis for susceility to disease in laboratory animals. Studies are aimed at improving rickettsial accines to prevent disease in troops operating in the field.  4. (U) 1. Mutagenization and selection of appropriate rickettsial strains by tissulture techniques. 2. Growth and purification of rickettsiae followed by polyacrylide gel electrophoresis of sub-cellular fractions. 3. Use of genetically homogeneous detectable mutants. The tissue culture technology developed, however, it engused to produce detectable mutants. The tissue culture technology developed, however, it engused for antigenic analysis of naturally occurring variants of rickettsiae. 2 inchemical studies of rickettsial macromolecules focused on isolation and identification employing polyacrylamide gel electrophoresis. Over 17 individual proteins were ecognized and molecular weight determinations conducted. 3. Over 30 inbred strains in feeding indicated resistance was dominant and coded for by one or a closely linked luster of autosomal genes. For technical report see Walter Reed Army Institute of luster of autosomal genes.	SPONSIBLE INDIVID	UAL			MAME. Osterman, Joseph V., Dr.						
Foreign intelligence not considered.  Revenue 202-576-3551  **Section of the control of the cont	AME: Rapmund	, Garrison, Co	OL, MC		телерном 202-576-2146						
Foreign intelligence not considered.  (U) Genetics; (U) Biochemistry; (U) Structure-Antigenicity  (U) 1. Genetic manipulation of rickettsiae to provide strains with optimum immunicity. 2. Isolation and purification of sub-cellular components responsible for liciting immunological protection. 3. Determination of the genetic basis for susceility to disease in laboratory animals. Studies are aimed at improving rickettsial accines to prevent disease in troops operating in the field.  4. (U) 1. Mutagenization and selection of appropriate rickettsial strains by tissulture techniques. 2. Growth and purification of rickettsiae followed by polyacrylide gel electrophoresis of sub-cellular fractions. 3. Use of genetically homogeneous more disease in the sais of rickettsial virulence.  5. (U) 76 10-77 09 1. Conditions for selection of temperature sensitive mutants of ickettsiae were established, but exhaustive mutagenesis with chemical agents faile o produce detectable mutants. The tissue culture technology developed, however, it is in the proving polyacrylamide gel electrophoresis. Over 17 individual proteins were ecognized and molecular weight determinations conducted. 3. Over 30 inbred strains dice were examined for resistance to rickettsial infection. Extensive cross and be reeding indicated resistance was dominant and coded for by one or a closely linked luster of autosomal genes. For technical report see Walter Reed Army Institute of	ELEPHONE 202-	576-3551			SOCIAL	SECURITY ACC					
Relationship: (U) Structure-Antigenicity 3. (U) 1. Genetic manipulation of rickettsiae to provide strains with optimum immunicity. 2. Isolation and purification of sub-cellular components responsible for eliciting immunological protection. 3. Determination of the genetic basis for suscendity to disease in laboratory animals. Studies are aimed at improving rickettsial raccines to prevent disease in troops operating in the field.  4. (U) 1. Mutagenization and selection of appropriate rickettsial strains by tissurative techniques. 2. Growth and purification of rickettsiae followed by polyacrylatide gel electrophoresis of sub-cellular fractions. 3. Use of genetically homogeneous indease to determine basis of rickettsial virulence.  5. (U) 76 10-77 09 1. Conditions for selection of temperature sensitive mutants of rickettsiae were established, but exhaustive mutagenesis with chemical agents failed to produce detectable mutants. The tissue culture technology developed, however, it is in the second produce of rickettsial macromolecules focused on isolation and identification employing polyacrylamide gel electrophoresis. Over 17 individual proteins were examined for resistance to rickettsial infection. Extensive cross and be recognized and molecular weight determinations conducted. 3. Over 30 inbred strains are decembered indicated resistance was dominant and coded for by one or a closely linked cluster of autosomal genes. For technical report see Walter Reed Army Institute of the second conducted and conducted and conducted and coded for by one or a closely linked cluster of autosomal genes. For technical report see Walter Reed Army Institute of the second code conducted and coded for successions.	GENERAL USE				ASSOCIA	TE INVESTIGATO	RS .	NAMES OF STREET			
Relationship: (U) Structure—Antigenicity 3. (U) 1. Genetic manipulation of rickettsiae to provide strains with optimum immunicity. 2. Isolation and purification of sub-cellular components responsible for eliciting immunological protection. 3. Determination of the genetic basis for suscendity to disease in laboratory animals. Studies are aimed at improving rickettsial vaccines to prevent disease in troops operating in the field.  14. (U) 1. Mutagenization and selection of appropriate rickettsial strains by tissual degree electrophoresis of sub-cellular fractions. 3. Use of genetically homogened in the genetic basis for suscending general degree electrophoresis of sub-cellular fractions. 3. Use of genetically homogened in the general degree established, but exhaustive mutagenesis with chemical agents failed to produce detectable mutants. The tissue culture technology developed, however, it is produced at the general studies of rickettsial macromolecules focused on isolation and identification employing polyacrylamide gel electrophoresis. Over 17 individual proteins were established and provided the produced and molecular weight determinations conducted. 3. Over 30 inbred strains and complete decreases and be recognized and molecular weight determinations conducted. 3. Over 30 inbred strains are provided indicated resistance was dominant and coded for by one or a closely linked eluster of autosomal genes. For technical report see Walter Reed Army Institute of the service of autosomal genes.	Foreign intelligence not considered.				HAME:	Robinson	, David,	LTC, VC	,		
Relationship: (U) Structure—Antigenicity 3. Technical Objective. 22 Approach, it Proceeds to Individual procession of the genetic basis for suscepticity in the process of the genetic basis for suscepticity to disease in laboratory animals. Studies are aimed at improving rickettsial vaccines to prevent disease in troops operating in the field.  24. (U) 1. Mutagenization and selection of appropriate rickettsial strains by tissurature techniques. 2. Growth and purification of rickettsiae followed by polyacrylande gel electrophoresis of sub-cellular fractions. 3. Use of genetically homogened inbred mice to determine basis of rickettsial virulence.  25. (U) 76 10-77 09 1. Conditions for selection of temperature sensitive mutants of rickettsiae were established, but exhaustive mutagenesis with chemical agents failed to produce detectable mutants. The tissue culture technology developed, however, it is produced at the process of rickettsial macromolecules focused on isolation and identification employing polyacrylande gel electrophoresis. Over 17 individual proteins were escapined for resistance to rickettsial infection. Extensive cross and be preeding indicated resistance was dominant and coded for by one or a closely linked eluster of autosomal genes. For technical report see Walter Reed Army Institute of cluster of autosomal genes.					NAME:	Oako St	anley.	GPT.	MEG		
3. (U) 1. Genetic manipulation of rickettsiae to provide strains with optimum immusencity. 2. Isolation and purification of sub-cellular components responsible for eliciting immunological protection. 3. Determination of the genetic basis for suscendity to disease in laboratory animals. Studies are aimed at improving rickettsial vaccines to prevent disease in troops operating in the field.  4. (U) 1. Mutagenization and selection of appropriate rickettsial strains by tissufficient techniques. 2. Growth and purification of rickettsiae followed by polyacrylatide gel electrophoresis of sub-cellular fractions. 3. Use of genetically homogeneously more disease were established, but exhaustive mutagenesis with chemical agents failed to produce detectable mutants. The tissue culture technology developed, however, it is produced at a subject of rickettsial macromolecules focused on isolation and identification employing polyacrylamide gel electrophoresis. Over 17 individual proteins were escognized and molecular weight determinations conducted. 3. Over 30 inbred strains and the entering indicated resistance was dominant and coded for by one or a closely linked cluster of autosomal genes. For technical report see Walter Reed Army Institute of cluster of autosomal genes.			(U)		(U) B	iochemis	try; (U)	Structi	ire-F	unction	
3. (U) 1. Genetic manipulation of rickettsiae to provide strains with optimum immusericity. 2. Isolation and purification of sub-cellular components responsible for eliciting immunological protection. 3. Determination of the genetic basis for suscenditity to disease in laboratory animals. Studies are aimed at improving rickettsial strains to prevent disease in troops operating in the field.  4. (U) 1. Mutagenization and selection of appropriate rickettsial strains by tissue techniques. 2. Growth and purification of rickettsiae followed by polyacrylande gel electrophoresis of sub-cellular fractions. 3. Use of genetically homogened in the disease in the disease of rickettsial virulence.  5. (U) 76 10-77 09 1. Conditions for selection of temperature sensitive mutants of rickettsiae were established, but exhaustive mutagenesis with chemical agents failed to produce detectable mutants. The tissue culture technology developed, however, is designed for antigenic analysis of naturally occurring variants of rickettsiae. 2 disochemical studies of rickettsial macromolecules focused on isolation and identification employing polyacrylamide gel electrophoresis. Over 17 individual proteins were examined for resistance to rickettsial infection. Extensive cross and be recognized and molecular weight determinations conducted. 3. Over 30 inbred strains are determined indicated resistance was dominant and coded for by one or a closely linked eluster of autosomal genes. For technical report see Walter Reed Army Institute of	Relationsh	ip: (U) Struct	ture-Antig	enicity	4-110-45		land of arch ===	baruelly Classics			
enicity. 2. Isolation and purification of sub-cellular components responsible for liciting immunological protection. 3. Determination of the genetic basis for susce ility to disease in laboratory animals. Studies are aimed at improving rickettsial accines to prevent disease in troops operating in the field. 4. (U) 1. Mutagenization and selection of appropriate rickettsial strains by tissulture techniques. 2. Growth and purification of rickettsiae followed by polyacrylide gel electrophoresis of sub-cellular fractions. 3. Use of genetically homogened by the dide gel electrophoresis of rickettsial virulence. 5. (U) 76 10-77 09 1. Conditions for selection of temperature sensitive mutants of cickettsiae were established, but exhaustive mutagenesis with chemical agents failed oproduce detectable mutants. The tissue culture technology developed, however, is eing used for antigenic analysis of naturally occurring variants of rickettsiae. 2 diochemical studies of rickettsial macromolecules focused on isolation and identification employing polyacrylamide gel electrophoresis. Over 17 individual proteins were ecognized and molecular weight determinations conducted. 3. Over 30 inbred strains side were examined for resistance to rickettsial infection. Extensive cross and be reeding indicated resistance was dominant and coded for by one or a closely linked luster of autosomal genes. For technical report see Walter Reed Army Institute of	3. (U) 1.	Genetic manin	ulation of								
diciting immunological protection. 3. Determination of the genetic basis for susceptility to disease in laboratory animals. Studies are aimed at improving rickettsial raccines to prevent disease in troops operating in the field.  4. (U) 1. Mutagenization and selection of appropriate rickettsial strains by tissufful to techniques. 2. Growth and purification of rickettsiae followed by polyacrylatide gel electrophoresis of sub-cellular fractions. 3. Use of genetically homogened in the disease of the											
dility to disease in laboratory animals. Studies are aimed at improving rickettsial vaccines to prevent disease in troops operating in the field.  4. (U) 1. Mutagenization and selection of appropriate rickettsial strains by tissuellure techniques. 2. Growth and purification of rickettsiae followed by polyacrylatide gel electrophoresis of sub-cellular fractions. 3. Use of genetically homogened in the model of the model											
accines to prevent disease in troops operating in the field. 4. (U) 1. Mutagenization and selection of appropriate rickettsial strains by tissual ture techniques. 2. Growth and purification of rickettsiae followed by polyacrylated gel electrophoresis of sub-cellular fractions. 3. Use of genetically homogened mice to determine basis of rickettsial virulence. 5. (U) 76 10-77 09 1. Conditions for selection of temperature sensitive mutants of ickettsiae were established, but exhaustive mutagenesis with chemical agents failed on produce detectable mutants. The tissue culture technology developed, however, if eing used for antigenic analysis of naturally occurring variants of rickettsiae. 2 iochemical studies of rickettsial macromolecules focused on isolation and identification employing polyacrylamide gel electrophoresis. Over 17 individual proteins were ecognized and molecular weight determinations conducted. 3. Over 30 inbred strains dice were examined for resistance to rickettsial infection. Extensive cross and be reeding indicated resistance was dominant and coded for by one or a closely linked luster of autosomal genes. For technical report see Walter Reed Army Institute of											
ulture techniques. 2. Growth and purification of rickettsiae followed by polyacrylade gel electrophoresis of sub-cellular fractions. 3. Use of genetically homogeneous noted mice to determine basis of rickettsial virulence. 5. (U) 76 10-77 09 1. Conditions for selection of temperature sensitive mutants of ickettsiae were established, but exhaustive mutagenesis with chemical agents failed of produce detectable mutants. The tissue culture technology developed, however, ageing used for antigenic analysis of naturally occurring variants of rickettsiae. 2 iochemical studies of rickettsial macromolecules focused on isolation and identification employing polyacrylamide gel electrophoresis. Over 17 individual proteins were ecognized and molecular weight determinations conducted. 3. Over 30 inbred strains like were examined for resistance to rickettsial infection. Extensive cross and be reeding indicated resistance was dominant and coded for by one or a closely linked luster of autosomal genes. For technical report see Walter Reed Army Institute of											
dide gel electrophoresis of sub-cellular fractions. 3. Use of genetically homogeneous notice to determine basis of rickettsial virulence.  5. (U) 76 10-77 09 1. Conditions for selection of temperature sensitive mutants of ickettsiae were established, but exhaustive mutagenesis with chemical agents failed of produce detectable mutants. The tissue culture technology developed, however, if eing used for antigenic analysis of naturally occurring variants of rickettsiae. 2 in individual proteins were experiently and molecular weight determinations conducted. 3. Over 30 inbred strains in its were examined for resistance to rickettsial infection. Extensive cross and be reeding indicated resistance was dominant and coded for by one or a closely linked luster of autosomal genes. For technical report see Walter Reed Army Institute of											
dide gel electrophoresis of sub-cellular fractions. 3. Use of genetically homogeneous notice to determine basis of rickettsial virulence.  5. (U) 76 10-77 09 1. Conditions for selection of temperature sensitive mutants of ickettsiae were established, but exhaustive mutagenesis with chemical agents failed of produce detectable mutants. The tissue culture technology developed, however, if eing used for antigenic analysis of naturally occurring variants of rickettsiae. 2 in individual proteins were experiently and molecular weight determinations conducted. 3. Over 30 inbred strains in its were examined for resistance to rickettsial infection. Extensive cross and be reeding indicated resistance was dominant and coded for by one or a closely linked luster of autosomal genes. For technical report see Walter Reed Army Institute of											
5. (U) 76 10-77 09 1. Conditions for selection of temperature sensitive mutants of ickettsiae were established, but exhaustive mutagenesis with chemical agents failed of produce detectable mutants. The tissue culture technology developed, however, is eing used for antigenic analysis of naturally occurring variants of rickettsiae. 2 inchemical studies of rickettsial macromolecules focused on isolation and identification employing polyacrylamide gel electrophoresis. Over 17 individual proteins were ecognized and molecular weight determinations conducted. 3. Over 30 inbred strains ice were examined for resistance to rickettsial infection. Extensive cross and be reeding indicated resistance was dominant and coded for by one or a closely linked luster of autosomal genes. For technical report see Walter Reed Army Institute of	ide gel ele	ectrophoresis	of sub-ce	llular frac	ctions	. 3. Use					
ickettsiae were established, but exhaustive mutagenesis with chemical agents failed oproduce detectable mutants. The tissue culture technology developed, however, it eing used for antigenic analysis of naturally occurring variants of rickettsiae. It is is included in the contemical studies of rickettsial macromolecules focused on isolation and identification employing polyacrylamide gel electrophoresis. Over 17 individual proteins were ecognized and molecular weight determinations conducted. 3. Over 30 inbred strains dice were examined for resistance to rickettsial infection. Extensive cross and be reeding indicated resistance was dominant and coded for by one or a closely linked luster of autosomal genes. For technical report see Walter Reed Army Institute of	nbred mice	to determine	basis of	rickettsia.	l viru	lence.					
o produce detectable mutants. The tissue culture technology developed, however, is eing used for antigenic analysis of naturally occurring variants of rickettsiae. It is is a considerable of the constant of	A STATE OF THE STA										
eing used for antigenic analysis of naturally occurring variants of rickettsiae. 2 iochemical studies of rickettsial macromolecules focused on isolation and identification employing polyacrylamide gel electrophoresis. Over 17 individual proteins were ecognized and molecular weight determinations conducted. 3. Over 30 inbred strains ice were examined for resistance to rickettsial infection. Extensive cross and be reeding indicated resistance was dominant and coded for by one or a closely linked luster of autosomal genes. For technical report see Walter Reed Army Institute of	ickettsiae	were establis	shed, but	exhaustive	mutag	enesis w	ith cher	mical age	ents	failed	
iochemical studies of rickettsial macromolecules focused on isolation and identification employing polyacrylamide gel electrophoresis. Over 17 individual proteins were ecognized and molecular weight determinations conducted. 3. Over 30 inbred strains ice were examined for resistance to rickettsial infection. Extensive cross and be reeding indicated resistance was dominant and coded for by one or a closely linked luster of autosomal genes. For technical report see Walter Reed Army Institute of											
ion employing polyacrylamide gel electrophoresis. Over 17 individual proteins wer ecognized and molecular weight determinations conducted. 3. Over 30 inbred strains ice were examined for resistance to rickettsial infection. Extensive cross and be reeding indicated resistance was dominant and coded for by one or a closely linked luster of autosomal genes. For technical report see Walter Reed Army Institute of											
ecognized and molecular weight determinations conducted. 3. Over 30 inbred strains ice were examined for resistance to rickettsial infection. Extensive cross and be reeding indicated resistance was dominant and coded for by one or a closely linked luster of autosomal genes. For technical report see Walter Reed Army Institute of											
ice were examined for resistance to rickettsial infection. Extensive cross and be reeding indicated resistance was dominant and coded for by one or a closely linked luster of autosomal genes. For technical report see Walter Reed Army Institute of											
reeding indicated resistance was dominant and coded for by one or a closely linked luster of autosomal genes. For technical report see Walter Reed Army Institute of	0		9								
luster of autosomal genes. For technical report see Walter Reed Army Institute of											
							er Reed	Army Ins	stitu	te of	
esearch Annual Progress Report, 1 Jul 76 - 30 Sep 77.	esearch Ani	nual Progress	Report, 1	Jul 76 - 1	30 Sep	77.					
6.5				65							

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 197 Molecular Biology of Rickettsia

Investigators.

Principal: Joseph V. Osterman, Ph.D.; LTC David Robinson, VC;

LTC Michael Groves, VC; CPT Stanley Oaks, Jr., MSC

Associate: Christine S. Eisemann; Janis Campbell; SP5 John Dewitt;

SP4 Fred DiLella

#### Description.

The genetic mechanisms underlying the interaction of rickettsiae and their hosts are being investigated to develop an understanding of virulence and antigenic variations. The approach includes use of inbred strains of mice to determine effect on virulence of rickettsiae and the development of a sensitive plaque assay technique for qualification of rickettsiae. Exploratory experiments have indicated this latter technique has the potential for discriminating between antigenic variants by the antibody mediated plaque reductions tests. In addition, biochemical studies have been directed toward purification of scrub typhus rickettsiae from contaminating host cell debris. Identification of peripheral macromolecules responsible for antigenic heterogeneity would be substantially aided by the preparation of highly purified R. tsutsugamushi.

#### Progress.

I. Genetics of natural resistance to scrub typhus injections in  $\operatorname{mice}$ .

The influence of genetic factors on the resistance of mice to bacterial and viral agents has been recognized, but little information exists concerning the inheritable resistance of mice to lethal rickettsial infection. Heaslip (1) suggested that stocks of white mice bred in Australia differed in their susceptibility to an isolate of Rickettsia tsutsugamushi, while other investigators reported that Swiss and Farm mouse stocks obtained in England differed in their susceptibility to the Imphal and Calcutta strains of scrub typhus rickettsiae. McDade and Gerone (2), reporting on the first successful plaque assay of R. tsutsugamushi, considered that the relationship between 50% mouse lethal dose (MLD<sub>50</sub>) and plaque titer could vary due to differences in susceptibility of Swiss and ICR mouse stocks. More recently, a brief study of several inbred strains of mice challenged with the Gilliam strain of R. tsutsugamushi indicated that the C57 Black mouse strain

differed from others in its heightened resistance to lethal rickettsial infection. These isolated, but consistent, observations suggested a relationship between the genetic background of mice and their resistance or susceptibility to lethal infection with  $\underline{R}$ . tsutsugamushi.

Evaluation of rickettsial virulence in mice has focused principally on rickettsial variants rather than strain differences in the animal host. Isolates of R. tsutsugamushi evidence different virulence characteristics in laboratory mice, and some investigators have suggested a direct correlation between severity of human scrub typhus infections and virulence of the etiologic strain for this laboratory host. Further extension of the concept suggested that rickettsial strains with reduced virulence for mice could be useful as attenuated strains for human immunization. R. tsutsugamushi, strain Gilliam, originally isolated from a patient with clinical scrub typhus and believed to have been attenuated by laboratory passage in embryonated eggs, was tested as an immunogen in Malaysian human volunteers. Despite its reduced virulence for stocks of laboratory mice, this rickettsial strain was found to cause clinical scrub typhus in man that was indistinguishable from naturally acquired disease. The widespread use of mice in laboratory studies, and the utilization of observed virulence characteristics as a basis for presumption of pathogenesis of infection in man and primate models, suggested to us the need for additional studies on the influence of genetic factors in resistance of mice to lethal infection with R. tsutsugamushi.

In this report, we present a survey of outbred mouse stocks and inbred mouse strains for susceptibility to  $\underline{R}$ .  $\underline{tsutsugamushi}$ , assess the resistance of  $F_1$  hybrids derived from inbred parents of both similar and different rickettsial susceptibilities, evaluate resistance of  $F_2$  and parental backcross generations of a selected inbred hybrid, and examine two possible mechanisms of resistance.

A. Survey of mice for response to  $\underline{R}$ .  $\underline{tsutsugamushi}$ , strains Gilliam and Karp.

The response of outbred mouse stocks to Gilliam infection varied. Six outbred stocks were tested, of which I was susceptible, 2 were resistant, and 3 were selectively resistant (Table 1). Stocks of outbred mice with a similar genetic origin, but obtained from different suppliers, showed little similarity in their response to Gilliam. Swiss mice were either susceptible or selectively resistant, and ICR mice were either resistant or selectively resistant depending on their source. Sex of the stock did not affect response pattern; both male and female Wrc:(ICR) mice were selectively resistant to Gilliam and evidenced similar resistance indices. All outbred mouse stocks were susceptible to Karp infection.

Table 1. Response of outbred mouse stocks to R. <u>tsutsugamushi</u>, strain Gilliam.

Outbred stock	Resistance index	Response pattern			
Swiss					
Mai:(S)	3	Susceptible			
Caw:CFW(SW)	40 Selectively resi				
Albino					
Caw:CF1	> 6.3 X 10 <sup>5</sup>	Resistant			
ICR					
Cr1:COBS CD-1(ICR)	$> 6.3 \times 10^{5}$	Resistant			
Dub: (ICR)	126	Selectively resistant			
Wrc:(ICR):male	320	Selectively resistant			
female	100	Selectively resistant			

Inoculation of inbred strains of mice with Gilliam resulted in a more uniform response to the rickettsiae. Mice were usually either susceptible (9 strains) or resistant (6 strains), and the erratic, selectively resistant response was observed only in the A/J strain (Table 2). Inbred mice derived from the same genetic line showed a similar response to Gilliam, regardless of their source. The four C3H and two DBA strains were all susceptible, and both BALB/c strains were resistant. No correlation was evident between response to Gilliam infection and known H-2 alleles of inbred mouse strains tested. All inbred strains of mice were susceptible to Karp infection.

# B. Response of $\mathbf{F}_1$ inbred hybrid mice to Gilliam and Karp.

Progeny from 5 different Gilliam-resistant by Gilliam-susceptible parental crosses were surveyed, as well as one hybrid cross (C3D2F $_1$ /J) derived from the mating of two susceptible parents. Two hybrids, C3CF $_1$ /Dub and CC3F $_1$ /Dub, represented reciprocal sexual crosses of C3H/HeDub X BALB/cDub mice. In the five F $_1$  hybrids of mixed parental susceptibility, Gilliam-resistance was dominant (Table 3). As expected, the Gilliam-susceptible parents produced susceptible progeny. Inoculation of male and female offspring with Gilliam indicated resistance was not sex linked, although resistant male F $_1$  mice usually survived ten-fold greater doses than did resistant females. All F $_1$  hybrids were susceptible to Karp infection.

Table 2. Response of inbred mouse strains to  $\underline{R}.$   $\underline{tsutsugamushi},$  strain Gilliam.

Inbred strain	Resistance index	H-2 Allele	Response pattern
A/HeJ	2	a	Susceptible
A/J	63	a	Selectively resistant
AKR/J	1.3 x 10 <sup>4</sup>	k	Resistant
BALB/cDub	$> 2.5 \times 10^4$	d	Resistant
BALB/cJ	$> 1.0 \times 10^4$	đ	Resistant
C3H/HeDub	3	k	Susceptible
C3H/HeJ	1	k	Susceptible
C3H/HeN	2	k	Susceptible
C3H/St	1	k	Susceptible
C57BL/6J	$> 3.2 \times 10^4$	ь	Resistant
C57L/J	$> 6.3 \times 10^5$	b	Resistant
CBA/J	1	k	Susceptible
DBA/1J	1	q	Susceptible
DBA/2J	1	d	Susceptible
SJL/J	1	s	Susceptible
SWR/J	3.2 x 10 <sup>4</sup>	q	Resistant

Table 3. Response of  $F_1$  inbred hybrid mice to Gilliam infection.

F <sub>1</sub> hybrid	Sex	Resistance index	Response pattern
AKD2F <sub>1</sub> /J: (AKR X DBA/2)	male	7.9 x 10 <sup>5</sup>	Resistant
(AKR X DBA/2)	female	7.9 x 10 <sup>4</sup>	Resistant
B6D2F <sub>1</sub> /J: (C57BL/6 X DBA/2)	male	1.3 x 10 <sup>5</sup>	Resistant
(C5/BL/6 X DBA/2)	female	1.3 x 10 <sup>4</sup>	Resistant
C3D2F <sub>1</sub> /J: (C3H/He X DBA/2)	male	1	Susceptible
(CSH/He X DBA/2)	female	3	Susceptible
LAF <sub>1</sub> /J: (C57L X A/He)	male	8.5 x 10 <sup>6</sup>	Resistant
(C3/L X A/He)	female	9.6 x 10 <sup>6</sup>	Resistant
C3CF <sub>1</sub> /Dub: (C3H/He X BALB/c)	male	$6.3 \times 10^6$	Resistant
(Con/He X BALB/C)	female	5.0 x 10 <sup>4</sup>	Resistant
CC3F <sub>1</sub> /Dub: (BALB/c X C3H/He)	male	1.0 x 10 <sup>6</sup>	Resistant
(BALB/C X C3H/He)	female	1.0 x 10 <sup>5</sup>	Resistant

C. Response to Gilliam infection of  ${\rm F_2}$  and parental backcross generations of C3H/HeDub X BALB/cDub mice.

In order to study the genetics of Gilliam resistance, large populations of  ${\rm F_2}$  and parental backcross  $({\rm F_1}$  X male parent) generations resulting from C3H/HeDub and BALB/cDub crosses were inoculated with 1,000 MLD $_{\rm 50}$  of Gilliam (as determined in C3H/HeDub mice). The percentage of each hybrid group surviving the infection closely approximated the survival rate expected, provided resistance followed simple Mendelian genetics, and acted as a single autosomal dominant trait (Table 4). The backcross of  ${\rm F_1}$  by susceptible parent yielded the expected ratio of one resistant to one susceptible; the progeny of  ${\rm F_1}$  by resistant parent were essentially all resistant, and the  ${\rm F_1}$  X  ${\rm F_1}$  cross  $({\rm F_2})$  produced the expected ratio of 3 resistant to 1 susceptible. Resistance was not linked to coat color.

Table 4. Response of  $F_2$  and parental backcross generations of C3H/HeDub X BALB/cDub mice to Gilliam infection.

		Percent survivors of					
Hybrid	Coat color	Gilliam in	fection a				
Backcrosses							
C3CF <sub>1</sub> X C3H/He	dark brown	47	(46/97) <sup>b</sup>				
CC3F <sub>1</sub> X BALB/c	white	96	(48/50)				
1	light brown	97	(28/29)				
	dark brown	100	(21/21)				
		Total 97	(97/100)				
F2CC3F1 X CC3F1	white	72	(18/25)				
2 1 1	light brown	84	(21/25)				
	dark brown	74	(35/47)				
		Total 76	(74/97)				

a 1,000 MLD<sub>50</sub> of Gilliam given I.P.; MLD<sub>50</sub> calculated in C3H/HeDub mice.

# $\,$ D. Infection of primary mouse embryo cell cultures with Gilliam.

The striking difference in susceptibility of C3H/HeDub and BALB/cDub mice to infection with Gilliam could result from a physiological inability of BALB/cDub cells to support the replication of these rickettsiae. To determine if Gilliam organisms could replicate in cells from both animals, embryo cell cultures of each type were infected under similar conditions, harvested after 5 days incubation, and rickettsial growth determined by microscopic observation of stained slides. No difference was observed; 34% of the C3H/HeDub cells were heavily infected compared to 38% of the BALB/cDub cells.

### E. Immunization of C3H/HeDub mice with Gilliam.

Differences in survival of mice after rickettsial infection could be related to host immunological deficiencies which preclude an effective immune response to a specific agent. Mice have been demonstrated to survive a potentially lethal dose of  $\underline{R}$ . tsutsugamushi when it is administered subcutaneously (s.c.), and animals are subsequently immune to lethal i.p. challenges. To determine if C3H/HeDub mice were capable

The numbers in parentheses indicate the ratio of surviving mice to total number of animals inoculated.

of mounting a protective immune response to the Gilliam strain, mice were inoculated s.c. with 1,000 or 10,000  $\rm MLD_{50}$  of Gilliam (as determined by i.p. inoculation of C3H/HeDub mice), and then challenged 28 days later by the i.p. route with 1,000  $\rm MLD_{50}$  of either Karp or Gilliam. Mice inoculated s.c. with either concentration of Gilliam survived the initial infection and were completely protected against subsequent homologous or heterologous challenge by the i.p. route.

F. Response of C3H/HeDub and BALB/c mice to other strains of  $\underline{R}.\ \underline{tsutsugamushi}.$ 

It was possible that C3H/HeDub mice were susceptible not only to the Gilliam strain, but to other strains of scrub typhus rickettsiae which elicited resistant or selectively resistant patterns in outbred mouse stocks. Strains TA678, TA686, and TA716, which produced a resistant response pattern in outbred Wrc:(ICR) mice and the Kostival strain, which produced a selectively resistant pattern, were used to infect C3H/HeDub mice. In addition, BALB/cDub mice, known to be resistant to Gilliam were tested with these same strains of rickettsiae. Table 5 shows that inbred mouse strains, unlike the outbred stock, were clearly differentiated in their response to Kostival; C3H/HeDub mice were susceptible and BALB/cDub were resistant. Infection of inbred mice with the other rickettsial strains elicited a response pattern similar to that observed in the outbred Wrc:(ICR) stock.

Table 5. Response of C3H/HeDub, BALB/cDub and Wrc:(CR) mice to selected strains of R. tsutsugamushi.

Rickettsial strain	Mouse	Resistance index	Response pattern
Kostival	C3H/HeDub	4 ,	Susceptible
	BALB/cDub	8.5 x 10 <sup>4</sup>	Resistant
	Wrc:(ICR)	130	Selectively resistant
TA678	C3H/HeDub	>6.3 x 10 <sup>5</sup>	Resistant
	BALB/cDub	>6.3 X 10 <sup>5</sup> >4.0 X 10 <sup>5</sup>	Resistant
	Wrc:(ICR)	$4.8 \times 10^{5}$	Resistant
TA686	C3H/HeDub	>1.0 x 10 <sup>8</sup>	Resistant
	BALB/cDub	>6.3 X 10 /	Resistant
	Wrc:(ICR)	$>1.8 \times 10^5$	Resistant
TA716	C3H/HeDub	2.0 x 10 <sup>6</sup>	Resistant
	BALB/cDub	4.0 X 10 <sup>5</sup>	Resistant
	Wrc: (ICR)	9.3 X 10 <sup>5</sup>	Resistant

The results of our studies clearly indicate that both outbred stocks and inbred strains of mice vary in their resistance to lethal infection with the Gilliam strain of  $\underline{R}$ .  $\underline{tsutsugamushi}$ . Inbred mouse strains that survived Gilliam or Kostival infection evidenced resistance indices as much as 6 X  $10^5$  times greater than those of susceptible mouse strains, but resistance was never absolute and could be overwhelmed by very large inocula of rickettsiae. Resistant and susceptible response patterns in inbred mice were both characterized by uniform, dose-related mortality of animals.

Random distribution of deaths and survivors throughout the titration range, which characterized the selectively resistant response pattern, was observed almost exclusively in outbred stocks of mice. The frequent observation of this erratic dose-response pattern in Gilliam infected Wrc:(ICR) mice originally led us to speculate that these mice might differ sufficiently in their genetic composition to affect natural resistance to infection. Survey of other outbred stocks from different suppliers indicated that 3 of the 6 stocks tested showed a similar pattern of selective resistance. The susceptible or resistant pattern observed in the 3 remaining outbred stocks was initially disconcerting, but it was considered possible that the production techniques of commercial breeders, employing closed colonies, could lead to some degree of genetic homogeneity.

Identification of the original breeding stock of outbred mice could not be correlated with the response pattern observed for the Gilliam strain of R. tsutsugamushi. A close correlation was observed with inbred mice, but this may have been influenced by the small number of strains tested and the common national origin of the commercial suppliers. Other investigators have reported that inbred CBA mice procured within the U.S.S.R. were resistant to Gilliam infection, but CBA/J mice included in this study were exquisitely susceptible to the Gilliam strain of R. tsutsugamushi maintained in our laboratory. Since the C3H/HeDub strain initially tested was found to be susceptible to Gilliam, 3 additional C3H strains were examined in an attempt to find a variant strain resistant to Gilliam which remained histocompatible with C3H/HeDub. The C3H/HeJ, C3H/HeN, and C3H/St mice, selected for their different responses to endotoxin, failed to show analogous differences in rickettsial susceptibility and all strains succumbed to Gilliam infection.

The  $\rm F_1$  progeny derived from crosses of inbred parents with different Gilliam susceptibilities were uniformly resistant to Gilliam infection regardless of sex. Only the cross of two sensitive strains yielded progeny susceptible to lethal Gilliam infection. Testing of  $\rm F_2$  and parental backcross generations of C3H/HeDub and BALB/cDub hybrids yielded percentages of resistant mice which also suggested that

resistance was genetically determined. It thus appeared that in the C3H-BALB/c system, resistance to Gilliam infection was dominant and was controlled by a single gene or closely linked cluster of genes which were autosomal and not linked to coat color. Furthermore, the survey of 16 inbred strains of mice did not indicate that resistance to Gilliam infection was associated with a particular H-2 allele.

The precise mechanism underlying susceptibility or resistance to lethal Gilliam infection was not determined. Susceptibility of C3H/HeDub mice was not the result of an inability to mount an immune response to Gilliam antigens, since subcutaneous infection was not lethal and did protect animals against subsequent intraperitoneal challenge with Gilliam or Karp. Also, resistance did not appear to be the result of a diminished capacity of Gilliam organisms to penetrate and multiply in BALB/cDub cells as compared to C3H/HeDub cells. Cultured cells from each strain, infected in vitro, evidenced similar levels of rickettsial growth. However, these results do not preclude differences in resistance mediated by immunocompetent cells of different mouse strains. Previous work both from this laboratory and another suggested that resistant strains of mice effectively suppressed Gilliam proliferation in the peritoneal cavity through evolution of rickettsiacidal macrophages.

The results of this study emphasize the importance of genetic differences among mouse strains in assessing the virulence of scrub typhus rickettsiae for this laboratory host. The Karp strain of R. tsutsugamushi was uniformly virulent for all inbred and outbred stocks tested, but substantial differences in susceptibility were evident among mouse strains inoculated with Gilliam or Kostival. Inoculation of a susceptible mouse strain (C3H/HeDub) with Karp, Gilliam or Kostival indicated no detectable difference in virulence and suggested that evaluation of rickettsial virulence in mice must be qualified by defining the genetic background of the host animal.

II. Plaque assay and cloning of scrub typhus rickettsiae in irradiated I-929 cells.

Plaque formation by rickettsiae, particularly the typhus and spotted fever group organisms, has been described in primary chick embryo cell culture and in continuous cell lines. In addition, optimum cultural conditions and diluents have been carefully examined using the primary chick embryo system. However, there has been little refinement of the plaque assay technique for scrub typhus rickettsiae since the initial report of successful plaquing in primary chick embryo cells (2). We investigated the ability of a continuous cell line to support plaque formation by  $\underline{R}$ .  $\underline{tsutsugamushi}$ , since this technique not only would provide a convenient plaquing procedure in urban laboratories, but would constitute a significant technical advance for overseas laboratories situated in remote geographical areas where

pathogen-free eggs are not routinely available. Previous work from this laboratory indicated that L-929 cells would support plaque formation by spotted fever group rickettsiae, and other investigators noted that plaque formation occurred in irradiated L-929 cells infected with typhus or spotted fever group rickettsiae. Since R. tsutsugamushi had been reported to grow in irradiated L-929 cell monolayers, it seemed reasonable to expect that plaque formation would occur. The purpose of this study were to: 1) determine cultural conditions for infected, irradiated L-929 cells which resulted in well-defined rickettsial plaques; 2) demonstrate the effectiveness of the plaquing system for classical scrub typhus strains and representative members of other rickettsial groups; and 3) devise a method for plaque purification and cloning of scrub typhus rickettsiae.

A. Effect of L cell concentration and incubation temperature on plaque formation.

These two variables first were examined using  $\underline{R}$ .  $\underline{conorii}$ , a spotted fever group organism capable of rapidly producing large plaques, then favorable conditions were tested with scrub typhus rickettsiae.

The density of L cell monolayers was controlled by adjusting the concentration of irradiated, non-replicating cells placed in culture dishes. After overnight incubation at 34C, each dish was infected with an appropriate dilution of R. conorii, overlayed and re-incubated at 34C. Neutral red staining overlay was applied on day 7 and on day 8 the plaques were photographed and measured. Table 6 indicates that an increase in cell density from 0.25  $\times$  10<sup>6</sup>/ml to 2.0  $\times$  10<sup>6</sup>/ml was accompanied by a two-fold reduction in plaque diameter. In each instance, plaques were symmetrical with well defined edges, although staining contrast was reduced at a cell density of 0.25 X 106/ml. The initial plaquing experiment with R. tsutsugamushi, strain Gilliam, was conducted at a cell concentration of 0.75  $\times$  10<sup>6</sup>/ml. Plaques were very small, usually less than 0.5 mm in diameter, and unsuitable for either plaque recovery or reliable enumeration. Only those cell concentrations favoring larger plaque diameter were suitable for further testing. Stydies conducted with cell concentrations of 0.5 X  $10^{6}/\text{ml}$  and 0.25 X  $10^{6}/\text{ml}$  indicated staining contrast was inadequate at the latter density. Plaques occurring in dishes seeded with 0.5 X  $10^6$  cells/ml were approximately 0.8 mm in diameter and clearly defined. This cell concentration was employed in all further experiments.

The effect of incubation temperature on plaque development also was studied first using  $\underline{R}$ .  $\underline{conorii}$  as the infecting organism. Adequate plaque formation was observed at 28, 30, 32, 34 and 36C. Temperatures exceeding 37C had a deleterious effect on the cell monolayers and insufficient stain was incorporated to provide adequate

Table 6. Effect of irradiated L-929 cell density on plaque size.

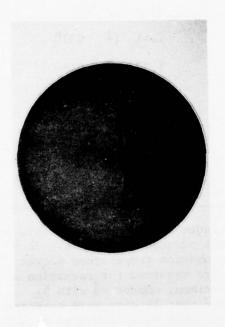
Number of L cells/ml (X 10 <sup>6</sup> )	Plaque diameter (mm)				
0.25	1.41 (± .03) <sup>a</sup>				
0.50	1.37 (± .04)				
0.75	1.21 ( <sup>±</sup> .03)				
1.00	0.95 (± .02)				
2.00	0.70 (± .03)				
andard error of the mean.					

contrast for plaque identification. Plaque development was rapid at 36C and well defined spotted fever group plaques were visible four days after infection. Reduction in incubation temperature slowed plaque formation and at 28C, 13 days were required for formation of well defined plaques. The initial experiment conducted with R. tsutsugamushi, strain Gilliam, indicated that 19 days were required for plaque formation when infected monolayers were incubated at 32C. This lengthy incubation period suggested that higher temperatures, favoring more rapid plaque development, should be investigated. Figure 1 illustrates that plaques formed by scrub typhus organisms at 34C were slightly larger and much more distinct than observed at 32C. Irradiated L cell monolayers did not remain viable after prolonged incubation at 36C.

Cultural conditions optimal for plaquing R. tsutsugamushi and acceptable for R. conorii included a cell concentration of 5 X 10<sup>3</sup>/ml and incubation at 34C. These conditions were employed in all further studies.

#### B. Effect of length of incubation.

The optimal time for plaque development and application of neutral red overlay was determined for all three classical strains of scrub typhus rickettsiae and for representatives of the spotted fever and typhus group organisms. A large number of dishes containing monolayers of irradiated L cells were infected with appropriate dilutions of each of the rickettsial strains and were then overlayed and incubated at 34C. Staining overlay was applied on successive days during the period when preliminary experiments had indicated that plaques



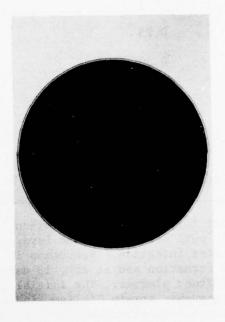


Fig. 1 (A)

Fig. 1 (B)

Figure 1. Effect of temperature on plaque formation by

R. tsutsugamushi, strain Gilliam. (A) Infected
L cell monolayer incubated at 32C for 19 days;

(B) Infected L cell monolayer incubated at 34C for 16 days.

were visible. The culture dishes were observed 24 hrs after application of the staining overlay to determine the presence and quality of plaque formation, as well as ease of enumeration. Table 7 indicates the first day that plaques were visible and also identifies the earliest day when plaque size and staining contrast were considered satisfactory for counting and plaque recovery. The scrub typhus group of rickettsiae required the longest incubation period, usually 19 days, although the Gilliam strain produced plaques in a noticeably shorter time than did the other two strains tested. The spotted fever group exhibited distinct, well defined plaques after only 5 days incubation. Plaques of the typhus group were small and indistinct after 12 days incubation, but neither continued incubation nor application of an intermediate feeder overlay on day 7 improved plaque morphology or clarity of staining. Figure 2 illustrates typical plaque formation by each group of rickettsia after the optimal length of incubation.

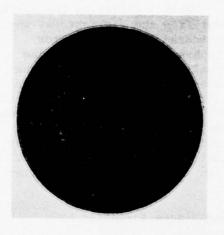
Table 7. Length of incubation required for plaque formation by rickettsiae in irradiated L-929 cells.

Rickettsiae	Day staining overlay applied				
R. <u>tsutsugamushi</u> , strain Karp	17, 18, <u>19</u> <sup>a</sup> , 20				
R. <u>tsutsugamushi</u> , strain Gilliam	15, <u>16</u> , 17, 18				
R. <u>tsutsugamushi</u> , strain Kato	17, 18, <u>19</u> , 20				
R. conorii	4, <u>5</u> , 6, 7, 8				
R. <u>rickettsii</u>	4, <u>5</u> , 6, 7, 8				
R. prowazekii	10, 11, <u>12</u> , 13				
R. typhi	10, 11, <u>12</u> , 13				

Underlining indicates earliest day on which plaque development and staining contrast were satisfactory for enumeration and plaque recovery.

The data in Table 8 suggest that the sensitivity of the two tests is similar. The plaque titer is similar to the MID and MLD values for Karp and Kato, which are lethal in small doses for this strain of mice. The Gilliam strain of R. tsutsugamushi, which is less virulent in ICR mice, evidences some disparity in titer between MLD and PFU, but the MID value is similar to the plaque titer.

Quantitative comparison of plaque assay and mouse assay.



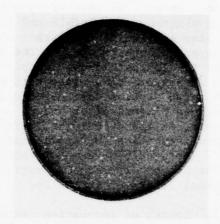
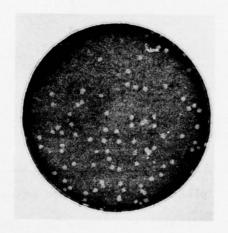


Fig. 2 (A)

Fig. 2 (B)



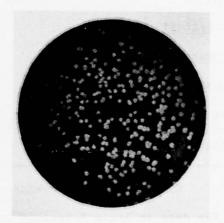
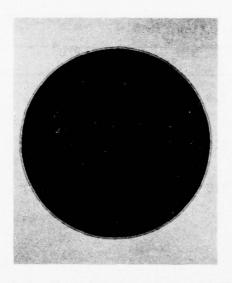


Fig. 2 (C)

Fig. 2 (D)



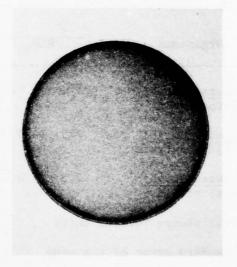


Fig. 2 (E)

Fig. 2 (F)

Figure 2. Plaque formation by scrub typhus group, spotted fever group and typhus group rickettsiae in irradiated L-929 cells. (A) R. tsutsugamushi, strain Karp; (B) R. tsutsugamushi, strain Kato; (C) R. conorii; (D) R. rickettsii; (E) R. prowazekii; (F) R. typhi

Table 8. Comparison of the plaque titers of scrub typhus rickettsiae with the MLD  $_{50}$  and MID  $_{50}$  titers in ICR mice.

		Ti				
Organisms	MLD <sub>50</sub>		MID 50		PFU	
R. tsutsugamushi Karp strain	7.3	(± 0.3) <sup>b</sup>	7.3	(± 0.3)	7.0	(± 0.1)
R. tsutsugamushi Gilliam strain	7.5	(± 0.5)	8.5	( <del>+</del> 0.3)	8.3	( <del>-</del> 0.1)
R. <u>tsutsugamushi</u> Kato strain	7.3	(± 0.3)	7.3	(± 0.3)	7.1	(± 0.1)

a PFU; plaque forming units

#### D. Cloning of rickettsiae.

Single plaques of scrub typhus rickettsiae were recovered with a sterile Pasteur pipette from monolayers evidencing only 1-10 plaques/ dish and were triturated vigorously in tubes containing 0.3 ml cold BHI broth. These suspensions were transferred to 35 mm dishes (35 X 10 mm, Lux, Microbiological Associates) containing monolayers of irradiated L cells at a concentration of 1.25 X 10° cells/dish, from which the growth medium had been removed. After adsorption for one hour at room temperature, fresh growth medium was added and the dishes reincubated at 34C. Monolayers were observed daily for signs of rickettsial infection (granulation, cytopathogenic effect, accumulation of detached cells in the medium) and an aliquot of cells was prepared for staining after 5-8 days incubation. Slides were fixed in methanol for 1 minute, rinsed, and stained with Giemsa stain (Harleco, Philadelphia, Pennsylvania) for 5 minutes. When a majority of the cells observed were heavily infected, all cells were resuspended in growth medium using a sterile teflon scraper and the infected cell suspension was transferred to a microchamber assembly (Dupont Co., Instrument Products Division, Rockville, Maryland) and blended 3 times at 45,000 RPM for 15 sec. An aliquot of this suspension was immediately diluted in ten-fold increments and replaqued using the method described above, while the remainder of the suspension was frozen at -70C. This procedure

b Standard error of the mean

was repeated 3 times, with each plaque recovery made from monolayers containing only 1-10 plaques. The final seed of plaque purified rickett-siae was propagated further in irradiated L cell monolayers to develop sufficient material for storage. This technique of plaque purification combined with terminal dilution was utilized to obtain clones of the three classical strains of scrub typhus.

Plaque assay of <u>R. tsutsugamushi</u> in chick embryo cells (2) resulted in small, indistinct plaques by all three strains after 17 days incubation. Irradiated, infected L cell monolayers required approximately the same incubation period for plaque development, but plaques were substantially more distinct. Previous studies comparing the sensitivity of the plaque assay in chick embryo cells and the mouse assay indicated that the plaque technique was considerably more sensitive than the mouse ID<sub>50</sub> procedure. However, the authors indicated through quotation of a personal communication (Elisberg) that the strain of mice employed in animal testing, or the physiological condition of the rickettsiae, may have affected their results. Our studies, using irradiated L cells for plaque titrations and ICR mice for animal titrations, suggest that the two techniques are very similar in sensitivity for detecting viable scrub typhus rickettsiae.

This investigation is, to our knowledge, the first report of plaque selection to enhance homogeneity of populations of scrub typhus rickettsiae. Such procedures are a necessary prerequisite for antigenic characterization of these organisms and may increase reliability of serological tests.

We have clearly demonstrated the usefulness of an irradiated continuous cell line in the plaquing and cloning of scrub typhus rickettsiae. In addition, L-929 cells support large, well defined plaque formation by representative members of the spotted fever group rickettsiae, but typhus group organisms produced small, indistinct plaques. The behavior of typhus group rickettsiae in irradiated L cells was interesting, because other investigators also have indicated the failure of  $\underline{R}$ . prowazekii to consistently form plaques in a mammalian cell line, although the organism is known to plaque reproducibly in chick embryo cells. The reciprocal relationship observed between L cell density and plaque diameter of  $\underline{R}$ . tsutsugamushi and  $\underline{R}$ . conorii also has been seen with chick embryo cells infected with R. typhi or R. rickettsii (4).

III. Isoelectric focusing of scrub typhus rickettsia.

Prior annual reports from this laboratory have alluded to the problem of purification of scrub typhus organisms. Last year's annual report lists a series of physical and chemical methods which have been tried and found unsatisfactory.

As an extension of this work we have tested the usefulness of isoelectric focusing for the separation of host material from scrub typhus organisms. This technique was originally described in 1961 (5) and

numerous papers since that time have attested to its usefulness. Intrinsically labelled rickettsial stocks were prepared as described in last year's annual report. Unlabelled stocks were prepared in L cell monolayers.

Initial experiments used columns prepared with a pH range of 3.5-10. When 150 lambda of an intrinsically labelled suspension of the Gilliam strain was focused for 48 hr on a 23 ml 0-56% sucrose gradient containing 1.8% ampholines, the label and the material which absorbed at 278 nm both focused in a 0.4 ml fraction near the acidic end of the column. This indicated that a better separation would be possible with a column having a narrower pH range in the acidic region. Therefore the ampholine composition was changed to 40% ampholine with a pH of 2.5-4.0, 40% with a pH of 4.0-6.0, and 20% with a pH of 3.5 to 10.0. The results of electrofocusing a portion of the same suspension in this composition of column is shown in Fig. 3. The radioactivity was centered in a narrow band at pH 3.5, but the OD peak did not completely coincide with the tritium peak. A significant amount of OD absorbing material was present in the 7 fractions obtained immediately following the 'H peak. Cold carrier focused coincident with the radioactive peak. A column run subsequently containing only sucrose and ampholine indicated that the OD absorbance in final 6 fractions was probably contributed by either the ampholine or the sucrose. OD readings on 56% sucrose solution indicated that the sucrose absorbed significantly at a wavelength of 278 nm.

The four fractions from the prior column with the highest radio-activity were pooled and refocused on a column containing 80% ampholines with a range of 2.5 to 4.0 and 20% with a range of 3.5 to 10. These data are shown in Fig. 4. The counts were spread over a broad area of the acidic region of the column and the early peaks of radioactivity were well separated from OD peaks.

Light and fluorescence microscopy of the peaks from these columns indicated that the peaks from the initial column contained large amounts of debris which stained with anti-L cell fluorescene isothiocyanate (FITC) labelled globulin. Fractions 33-35 of the second column contained many rickettsia-like bodies and little if any debris. Unfortunately, these organisms stained with the FITC labelled anti-L cell globulin as well as with labelled anti-rickettsial globulin. This indicated that the rickettsia were closely associated with host cell material.

To determine whether this L-cell material would focus at a different pH if it was freed from the rickettsia, L-cells were grown in the presence of  $^{14}\mathrm{C}$  amino acids, and given the same crude purification as the rickettsial pool. These L-cell fragments (no intact cells were seen on

light microscopy) were electrofocused in a 80% pH 2.5-4.0, 20% pH 3.5-10.0 gradient (Fig. 5). The peaks of radioactivity and OD coincided at a pH of 3.3. This was close to the pH of 3.5 found when rickettsia of L-cell origin were electrofocused. To further examine the relationship of these two preparations they were coisoelectrofocused. The results are shown in Fig. 6. As can be seen the peak counts in each preparation occurred in the same fractions.

We felt that separation of the L-cell material from the rickettsia would be impossible without altering the isoelectric points of the materials. Urea has been used to dissociate weakly bound protein complexes and thereby alter their physical properties. If these altered molecules exposed different sites the pH of the molecules might change. Fig. 7 represents the same L-cell and Gilliam preparations electrofocused in a 80% pH 2.5-4.0 and 20% pH 3.5-10.0 ampholine column which was poured with a 0-56% sucrose gradient and a 2-4 M urea gradient. The peaks coincide but occur at a pH of 3.8. The urea concentration was increased to 8M throughout the column without appreciable effecting the results. As can be seen by comparing Fig. 6 with Fig. 7 the presence of urea increased the apparent isoelectric point of the major peak of radioactivity from pH 3.0 to 3.8.

Triton X-100 is one of a series of nonionic detergents which have been used to fractionate biological materials. For this reason it was incorporated into a series of columns containing 2-4 M urea gradients.

Since Triton has an absorbance spectrum closely approximating that of protein (not shown), no worthwhile data was obtained from the OD reading of Triton containing columns. Both 0.2% and 1.0% concentrations were used with equivalent results. Fig. 8 shows the results of a column containing 0.2% Triton. The isoelectric point of the material containing the radioactive label was at pH 4.0 which was a modest increase from the pH of 3.8 seen in several columns containing 2-4 M urea only.

If other cell substrates suitable for the growth of rickettsia have peaks of radioactivity at pH values widely separated from the Gilliam strain, the rickettsia and host material might be more easily separated. To investigate this possibility radioactivity labelled Vero, HeLa, and LLC/MK2 cells were focused on columns with urea, urea and Triton, and on columns containing only ampholine. The results were analogous to those previously described i.e. Urea tended to increase the observed pH values but additionally it decreased the number of peaks. Triton had little observable effect other than the absorbance increase previously mentioned. For these reasons only representative results will be shown. In a column without Triton or urea, Vero cells had a peak of radioactivity at pH 3.3 (Fig. 9). HeLa cells had two peaks at pH's of 4.0 and 3.3 (Fig. 10). In a column containing 1% Triton, LLC/MK2 cells had radioactivity peaks at pH's of 3.3 and 3.6 (Fig. 11). From these data it

was arbitrarily decided to coisoelectric focus HeLa cell material and the standard radioactive labelled Gilliam suspension. The results are shown in Fig. 12. The major peaks of radioactivity coincided at a pH of 4.1, and the largest minor peaks at a pH of 3.8.

From these data we have concluded that the purification of scrub typhus from host components in isoelectric focusing columns with or without urea or urea plus Triton was not possible. The apparent isoelectric point of the organism was very close to that of the major cell components of the mammalian cells. It is possible that the rickettsia are intimately coated with a membrane of host cell material at the time they are released from the cell. Higashi (6) published electron micrographs which suggest this. If this is the case the chances of purifying scrub organisms free of host cell material by any mechanical or gentle chemical method seems remote. However, the coisoelectrophoresis experiments presented here indicate that there is an additional difficulty in the attachment of host cell material to rickettsia. In coisoelectric focusing we never saw a radioactive peak of rickettsia which was not coincidental with a peak of cell protein. Even when the materials are mixed in the column they solidly attach to each other and are not separated.

No further experiments involving isoelectric focusing for the separation of intact rickettsia from host cell material are planned.

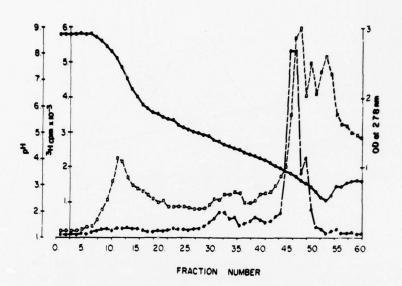


Figure 3. Isoelectric focusing of semipurified preparation of the Gilliam strain of R. tsutsugamushi in a 23 ml column containing 1.8% ampholines pH 2.5 - 4.0,40%, pH 4.0-6.0, 40% and pH 3.5-10.0, 20%. Electrofocusing was carried out at 4C with the voltage controlled at 700 V for 48 hr. The column was then fractionated in 0.4 ml amounts. 0--0 pH;  $\square$ -- $\square$  OD at 278 nm;  $\bullet$ -- $\bullet$  tritium counts;  $\Delta$ -- $\Delta$  14C counts. These are used on all remaining figures. The Gilliam strain was tritium labelled in all of the experiments.

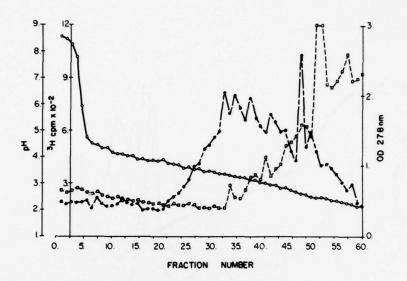


Figure 4. Isoelectric focusing of fractions 45-49 of the column represented in Fig. 3. Ampholine consisted of 80% with a pH of 2.5-4.0 and 20% with a pH of 3.5-10.0. Other experimental conditions constant.

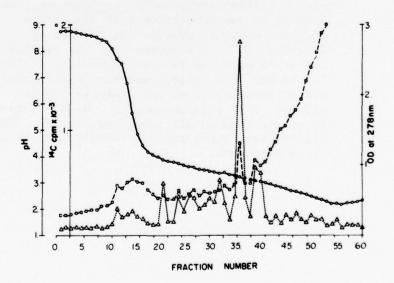


Figure 5. Isoelectric focusing of a semipurified preparation of L-cells. Experimental conditions were the same as Fig. 4.

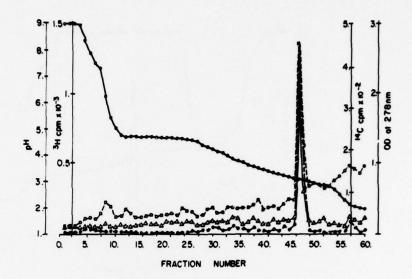


Figure 6. Coisoelectric focusing of the Gilliam strain and a L-cell preparation. Experimental conditions were the same as Fig. 4.

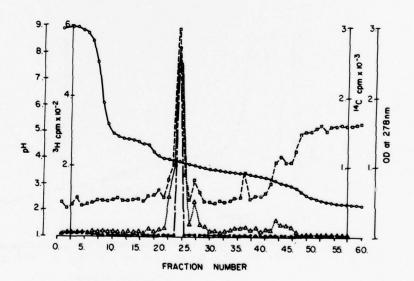


Figure 7. Coisoelectric focusing of the Gilliam strain and a L-cell preparation in a column containing a 2-4 M urea gradient. Other experimental conditions were the same as Fig. 4.

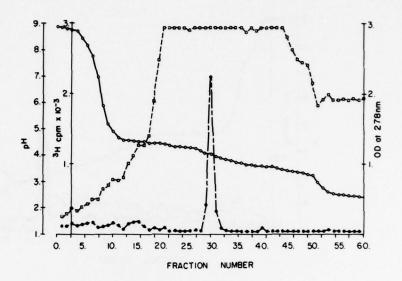


Figure 8. Isoelectric focusing of the Gilliam strain in a column containing a 2-4 M urea gradient and 1% Triton X-100.

Other experimental conditions were the same as Fig. 4.

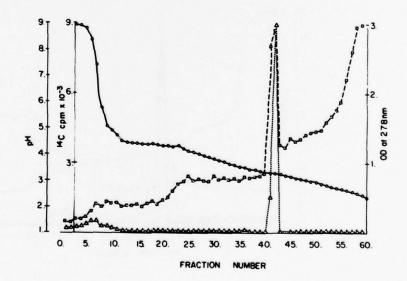


Figure 9. Isoelectric focusing of a Vero cell preparation. Experimental conditions were the same as Fig. 4.

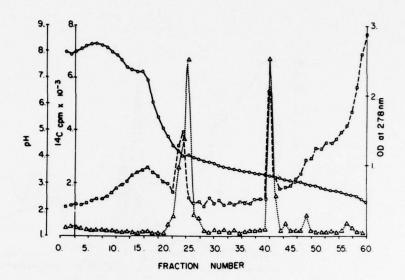


Figure 10. Isoelectric focusing of a HeLa cell preparation. Experimental conditions were the same as Fig. 4.

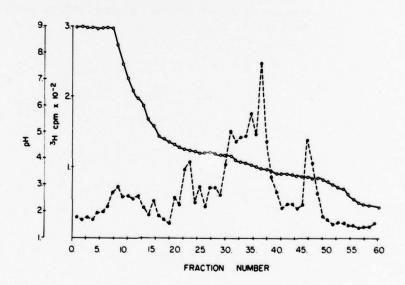


Figure 11. Isoelectric focusing of a LLC/MK<sub>2</sub> cell preparation in a column containing 1% Triton X-100. All OD readings were greater than 3.0. Other experimental conditions were the same as Fig. 4.

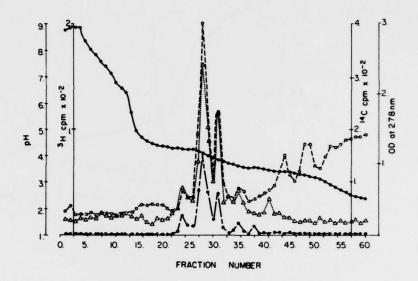


Figure 12. Coisoelectric focusing of the Gilliam strain and a HeLa cell preparation in a column containing 2-4 M urea. Other experimental conditions were the same as Fig. 4.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 197 Molecular Biology of Rickettsia

#### Literature Cited:

### References:

- 1. Heaslip, W.G. Tsutsugamushi fever in North Queensland, Australia. Med. J. Australia  $\underline{1}$ : 380-392, 1941.
- 2. McDade, J.E., and Gerone, P.J. Plaque assay for Q fever and scrub tyhus rickettsiae. Appl. Microbiol. 19: 963-965, 1970.
- 3. Weiss, E., Green, A.E., Grays, R., and Newman, L.M. Metabolism of <u>Rickettsia tsutsugamushi</u> and <u>Rickettsia rickettsi</u> in irradiated host cells. Infect. Immun. 8: 4-7, 1973.
- 4. Wike, D.A., Tallent, G., Peacock, M.G., and Ormsbee, R.A. Studies of the rickettsial plaque assay technique. Infect. Immun. 5: 715-722, 1972.
- 5. Svensson, H. Isoelectric fractionation, analysis and characterization of ampholytes in normal pH gradients. Acta Chem. Scand. 15: 325-341, 1961.
- 6. Higashi, N. Recent advances in electron microscope studies on ultrastructure of rickettsia. Zentral blatt fur Bakteriologie Parasitenkunde, Infektionskrankheiten und Hygiene 206: 277-283, 1968.

# Publications:

- 1. Eisemann, C.S., and Osterman, J.V. Proteins of typhus and spotted fever group rickettsiae. Infect. Immun. 14: 155-162, 1976.
- 2. Oaks, S.C., Jr., Osterman, J.V., and Hetrick, F.M. Plaque assay and cloning of scrub typhus rickettsiae in irradiated L-929 cells. J. Clin. Microbiol. 6: 76-80, 1977.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY					ACCES		2. DATE OF SUMMARY			CONTROL SYMBOL R&E(AR)636
1 DATE PREV SUM'RY	4. KIND OF SUMMARY	S. SUMMARY SCTY	& WORK SECURITY	7. REGR	AOC 6	04 DIS	77 09	SO SPECIFIC D	ATA-	S. LEVEL OF SUM
76 10 01	K. Completi	on II	11		NA		NL	TYES [	HO	A WORK UNIT
10. NO./CODES:*	PROGRAM ELEMENT		NUMBER		AREA NUM	MBER		WORK UNIT	_	
& PRIMARY	61101A	3A161101	A91C	0	0		20	00		
b. CONTRIBUTING										
c. CONTRIBUTING										
11. TITLE (Procede with	Security Classification Code	Physiolog	ic response	s of	the	cardi	ovascul	ar and n	erir	heral
Physiologic responses of the cardiovascular and peripheral  muscular systems to resuscitation 12 SCIENTIFIC AND TECHNOLOGICAL AREAS										
003500 Clin	ical Medicine	14. ESTIMATED COM	PLETION DATE	18 FUN	DING AGEN	icy		16. PERFORMA	HCE ME	THOD
74 07		7709		DA	1		1	C. In-		
17. CONTRACT/GRANT		1105		-	OURCES ES	STIMATE		OHAL MAN YRS	_	HD6 (In thousands)
& DATES/EFFECTIVE:	NA	EXPIRATION:		-	PRECEDIA		1	OHAL MAN THS	+	100 (// 2000
& NUMBER:				FISCAL	7/	6		,	1	91
G TYPE:		& AMOUNT:		YEAR	CURRENT		1-		1	- 21
& KIND OF AWARD:		f. CUM. AMT.			7	7	1		1	91
19. RESPONSIBLE DOD	REAPIZATION		T	20. PER	PORMINGO	RGANIZA	TION	1		7
NAME: Walter	Reed Army Ins	titute of	Research	HAME	alter	Reed	d Army 1	netitute	of	Research
							f Surge		. 01	Research
ADDRESS: Wash	ington, D.C.	20012					on, D.C.			
							,			
				PRINCIPAL INVESTIGATOR (Furnish SEAN II U.S. Academic Institution)						
RESPONSIBLE INDIVIDU	AL			MAME: Fleming, LTC, A.W.						
NAME: Rapmun	d, COL, G.			TELEPHONE: (202)576-3791						
TELEPHONE (202)	576-3551			SOCIAL SECURITY ACCOUNT NUMBER:						
21. GENERAL USE				ASSOCIA	TE INVEST	TIGATOR				E PAGE OF
				NAME:	Bell:	amy,	COL, R.			
Foreign int	elligence not	considere	d	NAME:						DA
22. KEYBORDS (Process)	EACH with Southly Classic	(U)	Myocardial	Meta	bolis	m; (1	U) Cardi	ac Dynan	nics	(U) Extra
Corporeal C	irculation; (	U) Tissue	ndividual paragrapho ide							
	elucidate the									
	hanges that o the coronary									
	s that frequen									
	anisms which									
modalities.	allisms whiteh	control co.	tollary liello	uynai	urcs c	courd	reau t	o better	Lie	atment
	dies have been	n designed	in a canin	e mod	to 1 wh	nich	can sel	ectively	det	ermine the
	f systemic pa									
	lood (blood									
	stem. Consci									
blood flow	under a varie	ty of condi	itions.							coronary
25 (U) 76 10	0 - 77 09. T	he influenc	ce of acid-	base	chang	es o	n mvoca	rdial fu	ncti	on has
been further										
been further defined. These studies demonstrate some of the differences in doing studies in the intact animal as opposed to isolated heart preparations. An acid pH is										
	detrimental to myocardial oxygen consumption and cardiac work in the intact animal.									
	nous pressure									
	ng the effect									
	ed by ventric									
	vessels perf					•				
	-					D		1 D.		- D
1 Jul 76 - 3	al report see 30 Sept 77.	warter Kee	ed Army Ins	citut	10 9.	Kese	arcn An	nual Pro	gres	s Keport
94										

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 200 Cardiac performance in by-pass surgery (Physiologic responses of the cardiovascular and peripheral muscular systems to resuscitation)

Investigators

Principal: LTC Arthur W. Fleming, MC Associate: COL Ronald F. Bellamy, MC

# I. The Influence of Systemic pH on Cardiac Hemodynamics.

- A. <u>Statement of the problem</u>. To determine the degree of redistribution of systemic and coronary blood flow, changes in systemic and myocardial oxygen consumption, and alterations in cardiac function that occur secondary to metabolic and respiratory acidosis and alkalosis.
- B. <u>Background</u>. Massive tissue trauma and hypotension which might be found in battle casualties are often associated with acidosis due to the accumulation of fixed anions in the blood. Acute ventilatory impairment may also occur in severe trauma cases and lead to a respiratory acidosis. Efforts at resuscitation may further exaggerate existing acid-base changes. Massive blood transfusions may initially lead to a systemic acidosis, followed by a systemic alkalosis due to citrate metabolism. The over-use of ventilatory support may lead to a respiratory alkalosis. The effects of severe acid-base changes on the heart are not completely understood.
- C. Experimental Approach. An extracorporeal circulatory system was devised to accomplish the following: The entire circulatory volume in an intact dog could be controlled on a minute to minute basis; the sympathoadrenal system could be maintained intact or separated from the coronary circulation; coronary blood flow and oxygen consumption could be easily measured without manipulation of the coronary arteries, and the heart rate and work could be controlled so that experimental animals were comparable to each other.
- D. Results and Discussion. When the pH was decreased to  $7.0 \pm 1$ , a marked increase in coronary blood flow occurred without a concommitant increase in oxygen consumption. Myocardial contractility progressively decreased with increasing acidosis. Systemic vascular resistance increased with acidosis, whereas coronary vascular resistance decreased at the same degree of acidosis. Alkalosis (pH  $7.55 \pm 0.07$ ) caused an increase in coronary blood flow with a concommitant increase in oxygen consumption and contractility. Systemic vascular resistance also decreased during alkalosis. It is postulated that acidosis interferes with the utilization of oxygen so that despite an increase in coronary blood flow, there is relative hypoxia of the myocardium. Although the myocardium was more stable with alkalosis, the relative benefits versus side-effects of alkalosis are yet to be determined.

E. <u>Conclusions and Recommendations</u>. Changes in systemic pH influence cardiac hemodynamics significantly. A considerable amount of investigative work is required, however, in defining the precise role of neurogenic factors and/or humoral factors.

# II. Muscle Surface pH

- A. <u>Statement of the Problem</u>. To evaluate muscle surface pH as an index of cardiac output, peripheral tissue perfusion and acid-base status.
- B. <u>Background</u>. A technique for measuring surface hydrogen ion activity (expressed as pH) is currently available. The assertion, however, that muscle surface pH is an index of tissue perfusion and systemic acid-base status has not been supported by actual measurements of blood flow to the involved muscle.
- C. Experimental Approach. A Swan-Ganz thermister catheter was inserted into the pulmonary artery by way of a jugular vein for measuring cardiac output. Femoral arterial blood flow was measured electromagnetically and the muscle surface pH of the same extremity was continuously monitored. Arterial and venous samples were also measured for pH, PO, and PCO,. Dogs were bled small increments (3-5%) of their estimated blood volume on multiple occasions while monitoring the parameters listed above.
- D. Results and Discussion. A decrease in muscle surface pH occurred 20-30 minutes before changes in systemic arterial pH and 5-10 minutes before venous pH changes when slow, progressive hemorrhaging was carried out. Technical problems with contamination of the surface of the pH probe with pooled (stagnant) blood has continued to be a problem and is further complicated by the loose skin of the canine model being used.
- E. <u>Conclusions and Recommendations</u>. The muscle surface pH probe still holds promise as a potential devise for routine monitoring of severely injured patients. Because of the loose skin in a dog, a different species of animal may be required. The bovine species is being considered as a potential model since the morphology of its skin more closely approximates that of humans.

#### III. The Calculation of Coronary Vascular Resistance

- A. <u>Statement of the Problem</u>. To reevaluate the classical method of calculating coronary vascular resistance in view of the concept of a vascular bed showing a critical closing pressure.
- B. <u>Background</u>. The calculation of vascular resistance (R) depends upon knowing the pressure gradient causing blood flow (F). The forward pressure is clearly arterial pressure (P) and the back pressure is usually taken to be the outflow or venous pressure (Pv). By the classical definition, the resistance that controls coronary flow is calculated as

- $(P-Pv)F^{-1}$ . In 1963 Permutt and Riley published a theoretical study of Burton's concept of the critical closure phenomenon in which they suggested that the classical definition would not be applicable to a vascular bed showing a critical closing pressure. Specifically they examined the question of how flow would be effected when vasomotor tone created a pressure collapsing the vessel that exceeded the outflow pressure. They showed that under such circumstances the relation between pressure and flow was such that flow stopped at a pressure  $(P_{f=0})$  determined by vasomotor tone and/or extramural compression. If this was true, resistance would be calculated as  $(P-P_{f=0})F^{-1}$ .
- C. Experimental Approach. A conscious instrumented dog model was used to compare the coronary vascular resistance calculated in the classical way (back pressure Pv) with resistance calculated with the assumption that flow is controlled by a different mechanism (back pressure  $P_{f=0}$ ). Resistance was calculated for resting flow and peak reactive hyperemia, the latter being taken as the minimal resistance of the vascular bed. Coronary vascular resistance changes during hemorrhagic shock were also studied.
- D. Results and Discussion. Resistance calculated as  $(P-Pv)F^{-1}$  increases during hemorrhage at the same time that resistance calculated as  $(P-Pf=0)F^{-1}$  is falling. The minimal resistance of the vascular bed (peak reactive hyperemia) calculated as  $(P-Pv)F^{-1}$  progressively increases if a proximal stenosis is present and becomes five times the value found in the unstenosed vascular bed. The minimal resistance calculated for the vascular bed as  $(P-P_{f=0})F^{-1}$  is not changed by the presence of a proximal stenosis.
- E. <u>Conclusions and Recommendations</u>. A critical evaluation of the theoretical basis of coronary vascular resistance is indicated in that these studies clarify the concept that the vascular resistance of a maximally dilated vascular bed <u>remains</u> low if a proximal stenosis is created, rather than increasing as was calculated by the classical formula (P-Pv)F-1.

# IV. Relationship of Diastolic Coronary Pressure to Flow Relations

- A. Statement of the Problem and Background. The relationship between pressure and flow is of fundamental importance in understanding the hemodynamics of a vascular bed. In comparison with other vascular beds, the relationship between pressure and flow in the coronary circulation is modified by two factors: (1) throttling of flow during each heart beat, and (2) predominate local control manifested in rapid autoregulation of flow to changing pressure. The current model offered a unique opportunity to study diastolic coronary pressure flow relations in a more physiologic state than previously reported.
- B. Experimental Approach. The conscious, chronically instrumented dog, in a semibasal state with a slow resting heart rate was used. Coronary

pressure flow relations were measured during the diastoles of individual beats in the resting state and then after flow was increased by reactive hyperemia and infusion of adenosine.

- C. <u>Results and Discussion</u>. Coronally pressure flow relations were described by a family of lines; diastolic flow being a linear function of aortic pressure. Zero flow pressure interrupts were estimated by extrapolation and found to vary between 20-55 mm Hg depending upon the magnitude of flow.<sup>1</sup>,<sup>2</sup>
- D. <u>Conclusions and Recommendations</u>. On the basis of these studies, when systolic pressures drops to 60-70 mm Hg and diastolic pressures of 40-50 mm Hg, coronary blood flow may be markedly diminished even in the presence of normal coronary arteries. Although mild degrees of hypotension are ususally thought to be tolerated fairly well, it is postulated that even short periods of hypotension may lead to irreversible ischemic changes. Further studies are required to confirm this.

# V. Development of a Practical Autologous Blood Transfusion Program

- A. <u>Statement of the Problem</u>. To develop a self-perpetuating method to help conserve national blood resources.
- B. <u>Background</u>. The need to conserve national blood resources even in peacetime is becoming increasingly apparent. The frequent low inventories following holidays in many cities and the drain on blood bank stores created by dramatic increases in the number and complexity of surgical procedures made it necessary to develop practical alternatives to existing practices of donor recruitment. Although only three percent of eligible blood donors in the United States donate blood, there is a large untapped reservoir of potential blood donors in the <u>patient population</u>. However, since a normal, healthy volunteer who is free of medical or surgical diseases can donate only one unit of blood every eight weeks, and must have a hematocrit of 41%, different criteria had to be established for patients.
- C. Experimental Approach. Every patient undergoing an elective surgical procedure was a potential candidate. A qualified surgeon discussed the concept of donating blood with each patient. If the patient was accepted into the program, an informed consent form had to be signed by the patient or his/her legal guardian. Blood was then donated and collected according to American Association of Blood Bank Standards and preserved as whole blood, fresh frozen plasma, packed cells or frozen packed cells.
- D. <u>Results and Discussion</u>. Patients donated one to 10 units of blood over a highly variable period of time. The freezing of red blood cells and plasma components made it possible to completely individualize phlebotomy schedules and offers a potential source for future conservation of national

blood resources. Patients who donated blood for their own operation markedly decreased the drain on blood bank stores and also reduced some of the logistical problems in obtaining certain blood types.4,5

E. Conclusions and Recommendations. Careful monitoring of high risk patients has demonstrated that most patients, even those with significant coronary artery disease, can safely predeposit most or all of the blood necessary for their own elective operation. The adoption of the techniques that we have outlined will add significantly to having larger reserves of blood products for any future military conflicts as well as for civilian emergencies.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 200 Cardiac performance in by-pass surgery (Physiologic responses of the cardiovascular and peripheral muscular systems to resuscitation)

#### Literature cited.

#### References:

- 1. Bellamy, R.F.: Diastolic coronary pressure flow relations in the dog. (In press)
- 2. Bellamy, R.F. and Lowensohn, H.S.: The effects of extravascular compression on coronary pressure flow relations. (Submitted)
- 3. Bellamy, R.F.: The calculation of coronary vascular resistance. (Submitted)
- 4. Fleming, A.W., Green, D.C., Radcliffe, J.H., St. James, D.M., and Fleming, E.W.: Development of a practical autologous blood transfusion program. (In press)
- 5. Fleming, A.W., Green, D.C. and Radcliffe, J.H.: Implementaion of a predeposit autologous blood transfusion program: A surgeon's viewpoint. (Submitted)

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				DAOC 6432		77 09 30		DD-DR&E(AR)636		
DATE PREV SUM'R	4. KIND OF SUMMARY	S. SUMMARY SCTY	S. WORK SECURITY	7. REGR	ADING" DA D		SA SPECIFI	C DATA-	S. LEVEL OF SU	
76 10 01	K. Complet	ion II			NA	NI.	W YES	□ mo	A WORK UNIT	
. NO./CODES:*	PROGRAM ELEMENT		NUMBER	TASK AREA NUMBER			WORK UNIT NUMBER		R	
PRIMARY	61101A	3A16110	1A91C	00		201				
. CONTRIBUTING										
. CONTRIBUTING										
	A Security Classification Code									
	olysis in Peri	pheral Blo	ood Vessels							
003500 C11	nical Medicine	14. ESTIMATED COM	PLETION DATE	TIS FUN	DING AGENCY		II. PERFOR	MANCE ME	THOD	
		W-100.00 (0.0000)		1	1	1				
73 07		77 09			OURCES ESTIMAT	-	L C.	In-H		
DATES/EFFECTIVE		EXPIRATION:		10. RES	PRECEDING	E A PROPER	BIONAL MAN Y	RS & FU	NDS (In thousands	
NUMBER:*	. MA	•		FISCAL	76		1		-0	
TYPE:		& AMOUNT:		YEAR	CURRENT	+		+	58	
KIND OF AWARD:		f. CUM. AMT			77		1		25	
RESPONSIBLE DOD	ORGANIZATION			20. PER	FORMING ORGANI	EATION	+		33	
we-#11-1	D 1 A T			┥						
walter	Reed Army Inst	itute of h	Research		Walter H			tute	of Resea	
DRESS:*				ADDRESS: Division of Surgery						
Washington, D.C. 20012				Washington, D.C. 20012						
				PRINCIP	AL INVESTIGATO	R (Fumish SEAN	II U.S. Academ	els Inelifution		
SPONSIBLE INDIVID	DUAL								•	
AME: Ranmun	d, COL, G.			MAME:* Buckman, MAJ, R.F. TELEPHONE: (202) 576-3796						
				SOCIAL SECURITY ACCOUNT NUMBER:						
TELEPHONE: (202) 576-3551				ASSOCIATE INVESTIGATORS						
				NAME:						
Foreign in	telligence not	considere	d	HAME:					DA	
E KEYWORDS (Proced	BACH with Socuelty Classifi	cetton Code) (U)	Fibrinolys	is; (	U) Wound	Healing	; (U)	Tendor	Repair	
(U) Ancrod										
TECHNICAL OBJEC	TIVE, 24 APPROACH, 28.	PROGRESS (Pumish	individual paragrapho id	lantified by	number. Precede t	at of each with	Socurity Closel	Reation Code	1.)	
23 (U) To	define the rol	e of fibri	n depositi	on an	d fibring	lysis i	n wound	heal	ing.	
tendon heal	ing, adhesion	formation	and vascul	ar th	rombosis.	Such	studies	may r	roduce	
	nto the genera									
	. Understandi									
	bidity and dis									
24 (U) The	effect of Bun	nel Figure	8 sutures	on t	he surfac	e plasm	inogen	activa	ator	
	f profunda ten									
subjected	to intrathecal	repair of	lacerated	digi	tal flexo	r tendo	ns. Ha	lf of	the	
animals re	ceived ancrod,	and the	other anim	als s	erved as	control	s.			
25 (U) 76	10 - 77 09. B	unnel sut	re is a po	tent	cause of	restric	tive te	ndon a	adhesion	
	be abandoned									
	ers little pro									
	following ten								3	
For techni	cal report see	Walter Re	ed Army In	stitu	te of Res	earch A	nnual P	rogres	s Repor	
	- 30 Sept 77.							- 0		
			1	01						

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 201 Fibrinolysis in peripheral blood vessels

Investigators

Principal: MAJ Robert F. Buckman, Jr., MC

#### Fibrinolysis - Tendon Wounds

Background and Statement of the Problem. The fibrinolytic activa-A. tor system is a cascading enzyme system which is found in tissues of mesothelial origin. Plasminogen, an inactive precursor molecule, is converted by activators contained within tissue and blood to plasmin, an active lytic endopeptidase which attacks and degrades fibrin and fibrinogen. This system is important in (1) maintanence of vascular patency by the removal of fibrin depositions which occur throughout daily activity and (2) in wound healing by molding and structuring the original fibrin deposits so that they can be absorbed and replaced by normal tissue. The absence of fibrinolysis in the vascular system is known to predispose to vascular thrombosis. Decreased levels of tissue activator may lead to hypertrophic wound healing and significant adhesion formation. Because of evidence that fibrin and fibrinolysis may have a role in both normal and pathological wound healing processes, studies of the healing of tendon wounds were made to determine whether fibrinolysis played a role in pathologic tendon adhesion formation.

Failure of tendon glide, caused by restrictive post-operative adhesions, continues to prohibit the primary repair of lacerated intrathecal digital flexor tendons, and constitutes a major unsolved problem in hand surgery. Although the pathogenesis of tendon adhesion formation is poorly understood, it is known that some adhesions are necessary to supply the vascular and cellular elements which heal tendon lacerations. The tendon itself possesses little, if any, intrinsic tenoblastic activity.

The effect of the most commonly-used technique of tendon repair was studied with reference to the mechanism of adhesion formation. Of particular interest was the effect of tendon sutures on the plasminogen activator system, which may be a regulator of local scarring.

B. Experimental Approach. Fibrinolysis has been strictly quantitated in this laboratory by the perfection of the fibrin slide and plate techniques of Astrup and associates. Fibrinolytic activator is quantitated by the placement of tissues or euglobulin extracts of tissues on fibrin plates, with determination of the subsequent zones of lysis after 18 hours incubation at 37°C. Plasminogen is differentiated from plasmin activity by utilizing plates which had been heated at 80° for 15 minutes. Fibrin slides are utilized with a semi-quantitative localization of fibrinolytic activator activity. For the first time, a technique for the identification of inhibitors of the fibrinolytic system has been developed in this laboratory. The technique consists of placing a biopsies of tissues suspected of possessing fibrinolytic inhibitors around a tissue biopsy possessing a known activator activity and measuring resultant zones of inhibition of lysis.

The effect of Bunnell Figure 8 sutures on the surface plasminogen activator activity of profunda tendon within the digital theca was studied, using the method described above. Serial histologic sections, specially stained for fibrin cells collagen were used to correlate in vitro plasminogen activator activity with in vivo healing.

C. <u>Results and Discussion</u>. Bunnell suture of an otherwise uninjured tendon was found to be associated with significant depression of local plasminogen activator activity, and with coagulation necrosis of the sutured area of tendon. Failure of the local plasminogen activator system is associated with persistence and eventual fibrous organization of fibrin locally deposited as the result of operation.

Bunnell suture is itself a potent cause of restrictive tendon adhesions, and probably should be abandoned in favor of available techniques less traumatic to the tendon.  $^{\rm l}$ 

#### II. Tendon Wounds

- A. <u>Background and Statement of the Problem</u>. The observation that repair of digital flexor tendons produced restrictive post-operative adhesions by causing failure of the local plasminogen activator system and allowing the persistence and organization of fibrinous exudates, led to an attempt to decrease adhesions by limiting local fibrin deposition.
- B. Experimental Approach. Rhesus monkeys were subjected to intrathecal repair of lacerated digital flexor tendons. Half the animals received the defibrinogenating agent ancrod post-operatively, in doses which maintained systemic afibrinogenemia for 96 hours after operation. Half of the animals received no ancrod. Three weekd post-operatively, work of flexion of operated fingers from ancrod-treated and control animals, and the breaking strength of tendon anastomoses from both groups, were measured on an Instron tensiometer. Serial histologic sections of operated fingers of treated and untreated animals were also obtained at 3 weeks.
- C. Results and Discussion. In operated fingers of ancrod-treated animals, work of flexion was significantly reduced compared to operated fingers of untreated controls. A significant decrease in tendon anastomosis breaking strength was also observed in ancrod-treated animals. Work of flexion and anastomotic breaking strength were found to be proportional, both in the treated and untreated groups analyzed separately and for the combined groups. Histology revealed that adhesions to the sutured region of tendons had not been completely prevented by the regimen of ancrod used, nor was evidence seen of pathologic hematoma-seroma formation in fingers of treated animals. It was concluded that short-term ancrod defibrinogenation reduced the adhesive restriction of tendon glide following tenorrhaphy, but that this effect was attended by a proportional reduction in the strength of tendon healing. The regimen of ancrod used in the present study appears to offer little promise of clinical usefulness,

in the prevention of the "no glide" phenomenon following tenorrhaphy. A Linear relationship appears to exist between the resistance to tendon glide, as measured by work of flexion, and anastomotic breaking strength, prior to digital mobilization. This relationship makes it likely that agents such as ancrod, which improve tendon glide after tenorrhaphy by non-selectively inhibiting scar formation, may not decrease resistance to glide without proportionally decreasing the strength of healing of tendon anastomoses.<sup>2</sup>

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 201 Fibrinolysis in peripheral blood vessels

#### Literature cited.

#### References:

- 1. Buckman, RF, Hufnagel, HV, Olivier, G, Buckman, PD, and Zuidema, GD: Some effects of Bunnel suture on otherwise uninjured tendons in subhuman primates. Surgery. Accepted for publication.
- 2. Buckman, RF, Woods, M, Bell, WR, Buckman, PD, and Zuidema, GD: Modification of post-operative tendon adhesions by ancrod defibrinogeneration. Surgery. Accepted for publication.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY					DA OC 6433		77 09 30		DD-DR&E(AR)636	
V	Completion	S. SUMMARY SCTY	6. WORK SECURITY	7. REGR	A A	NI NI	CONTRACTO	C DATA-	S. LEVEL OF SUM	
76 10 01	OGRAM ELEMENT		NUMBER		REA NUMBER			IT NUMBER		
Page 100 to 10 to	51101A	3A161101A		00		202			-	
b. CONTRIBUTING	DITOIA	SAIGITOTA	210							
c, CONTRIBUTING										
	genic Compos	sition of	Trypanosome	s						
002600 B		100 Microb								
74 07		14. ESTIMATED COM	77 09	DA C. In-Hol						
17. CONTRACT/GRANT				16. RES	DURCES ESTIMA	-	ROFESSIONAL MAN	RS & FUI	b. FUNDS (In thousands)	
A DATES/EFFECTIVE: NA		EXPIRATION:			76		4.0		74.	
b. NUMBER:*		d AMOUNT:		FISCAL	CURRENT	-		-	- , - ,	
C. TYPE:		f. CUM. AMT			77		4.0		135	
19. RESPONSIBLE DOD ORGA	ANIZATION	J. COM. AMT		20. PER	FORMING ORGA	MOITASIN	————		T	
MAME: Walter Reed Army Institute of Research ADDITION OF COMI ADDITION OF							f Research			
RESPONSIBLE INDIVIDUAL  HAME: Garrison Rapmund, COL  YELEPHONE: 202-576-3551  21. GENERAL USE  Foreign intelligence not considered					PRINCIPAL INVESTIGATOR (Pumbeh SEAN II U.S. Academic Institution)  NAME:  Carter L. Diggs  TELEPHONE: 202-576-3544  SOCIAL SECURITY ACCOUNT NUMBER:  ASSOCIATE INVESTIGATORS  NAME:					
	•			NAME:						
(U) Protozoa;	(U) Tropic	cal_Medici	Immunity; ne: (U) Ant	(U) A ibodi	ntigens es	; (U)	African t	rypano	somiasis;	
23 (U) The converse of the con	objective of the host res for induction axis agains approach is of the effects. Emphasi	f this work sponse to the on of immun it these min to develop its of immun s is on ar	c unit is to the trypano nity, and to ilitarily in systems in the stimular of the stimular tripular tr	o cha somes o inv mport n exp	racteri , to ch estigat ant dis eriment	aractore the	erize the feasibili . imals and	antige ty of in cul	ture for	
25 (U) 76 10-77 09 In vitro studies of the interaction of antibody with Trypanosoma rhodesiense indicate the participation of normal splenocytes in non complement dependent killing. Multiple antigenic types of a recent human isolate have been obtained in clones; antigen has been extracted from each and specific antibody prepared. This line of research is being transferred to work unit 083. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1976 - 30 September 1977.										
			105							

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH Work Unit 202 Antigenic composition of trypanosomes

Investigators: COL Carter L. Diggs, MC, M.D., CPT Gary H. Campbell MSC, Ph.D. John Barbaro, Ph.D.; D.T.O. Wong, Ph.D. MAJ Ray Perry, MSC, Ph.D., and MAJ Richard MacDermott, MC, M.D.

Assistants: Andre J. Toussaint, M.S.; Barbara J. Flemmings, B.S; James Dillon, B.S., Klaus Esser, B.S., John Bussey, Chris Goodhue, B.A., Ruta Hajkowski, B.S.; SP6 Barry Ellis, B.S., SP4 Karen Czarnecky, B.S., and Maurice Schoenbechler, B.S.

- 1. The Transfer of Protection against <u>Trypanosoma rhodesiense</u> by specific mouse spleen cell populations.
- A. Objective: The objective of these experiments was to investigate some aspects of protection to T. rhodesiense infection transferred with unfractionated immune mouse spleen cells from hyperimmune donors and with specifically enriched spleen cell populations.
- B. Description: Inbred C57BL/6J mice were hyperimmunized by a regimen of five weekly injections each consisting of 1 x 10 gamma irradiated T. rhodesiense (Wellcome strain) per mouse. Immune spleen cells were taken from these mice 7 days after the last injection. For some experiments immune spleen cells were separated by adherence to plastic and characterized by particle latex phagocytosis.

Non-plastic adherent fractions were treated by iron carbonyl and density centrifugation before fractionation into B- and T- enriched cell populations by immunoabsorbent column chromatography. Cells were characterized by the presence of surface immunoglobulin.

C. <u>Progress</u>: Mice receiving five innoculations of 1 x 10<sup>7</sup> irradiated trypanosomes were immune to challenge infections of 200 trypanosomes. Spleen cells from these hyperimmune mice were used for the following studies:

The first experiments were designed to determine if immune spleen cells, could protect recipient mice against trypanosome infecttion and how many spleen cells were needed to protect. As shown in figure 1 at least 25 x 100 immune spleen cells were needed to protect. Normal spleen cells did not protect. Mice in these first experiments were challenged with trypanosomes seven days after receiving

cells. In the next series of experiments the time course of protection transferred by immune spleen cells was determined. More specifically the questions of how soon after receiving immune cells can protection be confirmed and how long after receiving immune cells does protection last were addressed. As shown in figure 2 mice that were infected one or two days before receiving 70 x 10<sup>6</sup> immune spleen cells died when challenged with 200 trypanosomes. Mice given immune spleen cells on the same day as receiving the trypanosome infection only lived two days longer than the control mice receiving normal spleen cells. However, when immune spleen cells were given one day prior to infection, the mice were protected from the trypanosome challenge. This protection was viable for 21 days after receiving the immune spleen cells.

The first two series of experiments showed that immune spleen cells, from hyperimmune mice that had received five injections of irradiated trypanosomes, protected recipient mice from a homologous trypanosome challenge. The next group of experiments were designed to describe the type of spleen cell responsible for transferring protection. Spleen cells were separated into macrophage enriched cell population, by adherence to plastic, and into T enriched cell populations and B-enriched cell populations by goat anti-mouse gamma globulin immunoabsorbent column chormatography.

Large numbers of macrophages were not obtainable by the experimental methods used. As indicated in figure 3, the transfer of protection by macrophages was only 50% successful when 7 x  $10^6$  cells were used. The question still remains unanswered as to whether more macrophages would convey protection.

Fewer B-enriched spleen cells were needed to transfer protection. As shown in figure 4, as few as 5 x  $10^6$  cells transferred solid immunity to trypanosome challenge infections. However, no protection was obtained when as many as 54 x  $10^6$  T-enriched spleen cells were transferred as indicated in figure 5.

<u>Discussion:</u> Spleen cells from mice made hyperimmune to  $\underline{T}$ . <u>rhodesiense</u> by 5 weekly injections of  $1 \times 10^{0}$  irradiated trypanosomes transferred protection to a trypanosome infection in all recipient mice. It appears that B-enriched spleen cell transfer the protection probably by the production of specific anti-trypanosomal antibody in the recipient mouse T-enriched spleen cells populations did not transfer protection. The partial protection observed by the transfer of  $7 \times 10^{0}$  macrophage enriched cells is interesting because if the transfer of protection was due to the B-cell contaminants this indicates that B-cells and macrophages transfer protection better than B-cells alone do. More studies are needed in this area.

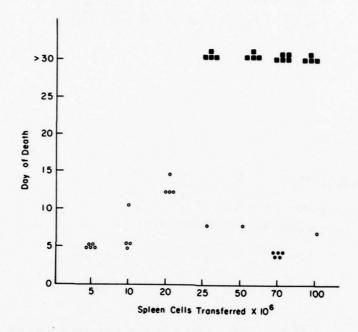


Figure 1

Spleen cells from mice receiving a regimen of five weekly injections of 1 x 10 gamma irradiated <u>T</u>. rhodesiense per mouse were transferred to recipient mice. Seven days after receiving the cells recipient mice were challenged with was 200 homologous trypanosomes. The day of death after challenge was recorded as: • death-immune cell recipients; • -alive immune cell recipient • -death-normal recipient.

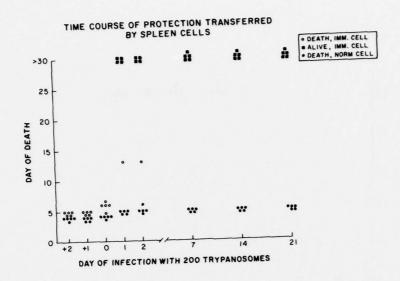


Figure 2

The time course of protection transferred by immune spleen cells as determined by giving 70 x 10 mmune spleen cells 1 or 2 days after infecting with 200 trypanosomes, giving the cells on the same day as the infection, or giving the cells 1,2,7, 14 or 21 days prior to infecting with trypanosomes. Protection to the trypanosome infection was observed when immune spleen cells were given one day prior to infecting. Protection was viable 21 days after receiving the cells.

# TRANSFER OF PROTECTION AGAINST T. RHODESIENSE BY MACROPHAGE ENRICHED SPLEEN CELLS

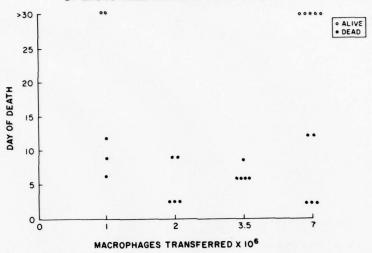
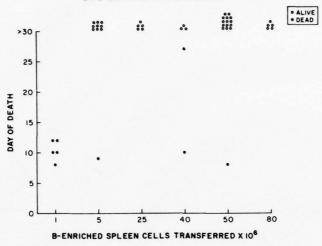


Figure 3

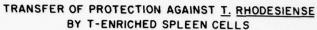
Macrophages were separated from immune spleen cells by adherence to plastic. Macrophages were 90-93% viable, 16-21% immunoglobulin positive 70-73% positive for latex particle ingestion. Low numbers of macrophages were recovered by the adherence to plastic method. The transfer of protection against  $\underline{\mathbf{T}}$ . rhodesiense by the macrophage enriched spleen cell population was only 50% successful when 7 x 106 cells were used.





#### Figure 4

B-enriched spleen cells were obtained by passing non-plastic adherent, iron carbonyl treated, density centrifugation treated spleen cells over a Sephadex G-200 immunoabsorbent column. Column adherent cells were eluted from the column by normal mouse serum. B-enriched spleen cells were 68-75% positive for surface immunoglobulin, 98.2% viable and less than 2% of the cells ingested latex particles. A minimum of  $5 \times 10^6$  B-enriched spleen cells was needed to transfer protection.



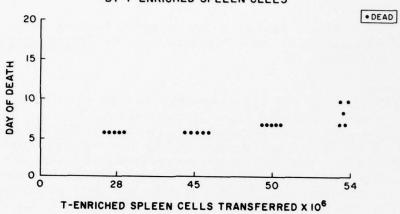


Figure 5

T-enriched spleen cell populations were obtained from non-plastic adherent spleen cells that were treated with iron carbonyl and density centriguation before being passed over an immunoabsorbent Sephadex G-200 column. The T- enriched spleen cells did not adhere to the column. These cells were 10% positive for surface immunoglobulins and were 86% viable.

II Mechanisms of Immunity in African trypanosomiasis

A. Objective: These studies were designed to help define the immune mechanisms involved in resistance to Trypanosoma rhodesiense infections.

B. Description:

Both antibody and cellular responses can be elicited by immunization or infection with African trypanosomes. In mice, resistance to T. rhodesiense and T. gambiense can be transferred with serum or B but not T cells from immune donors to normal recipients. Congenitally athymic (nude) mice are as resistant as littermate controls to infection with T. rhodesiense and can be effectively immunized. These observations suggest that B cells and their products are important in the development of resistance in mice, to infection with trypanosomes. However, a contribution of T lymphocyte effector mechanisms in this resistance cannot be excluded. In mice, T lymphocyte sensitization to trypanosome antigens has been demonstrated by both assays for in vitro blastogenesis in response to trypanosome antigen and delayed type hypersensitivity (footpad swelling).

In order to determine if T cells could have an effector role in the absence of antibody during infection with T. rhodesiense, B cell deprived mice were used. These mice were treated from birth with goat antibody to mouse- $\mu$  chain ( $\mu$  suppression) to antigens, gens, while leaving allograft rejection, helper T cell function, GVH reactivity, and in vitro T cell mitogen and mixed lymphocyte culture responses intact. Also, an adoptive spleen cell transfer system was used to determine the relative T and B cell contribution to the long term resistance to challenge seen in immunized animals.

#### C. Progress:

- 1. Studies on the Development of Resistance to T. rhodesiense.
- a. Course of infection in control or  $\mu$ -suppressed mice. Five  $\mu$  suppressed male C57Bl/6J mice and five age and sex matched control mice injected from birth with NGS (globulin Fraction) were infected by i.p. injection of  $10^6$  T. rhodesiense organisms. The results (Fig. 6) indicated a significant (p < .01, Student's t-test) increase in susceptibility of  $\mu$  suppressed mice to infection with trypanosomes The mean time of death for the  $\mu$  -suppressed mice as 14.6 + 1.6 (S.E.) days or compared to 31.8 + 3.4 days for control mice. Although the rate of appearance of parasites in the  $\mu$ -suppressed mice was delayed, the mice failed to reduce the peak of parasites and died after a persistently high (3.5 X  $10^6$  organisms/ml) parasitemia.

In contrast, all control mice reduced the first peak of parasitemias to undetectable levels (<10 organisms/ml), but succumbed to subsequent waves of parasitemia.

b. Course of infection with T. rhodesiense in mice exposed to 900  $\frac{R\ before\ infection.}{R\ before\ infection.}$  If  $\mu\text{-suppressed}$  mice were completely deficient in immunity to trypanosomes and the growth of the organisms was not limited by other factors, the parasites might have been expected to increase logrithmically until the day of death without the plateau of parasitemia which occurred beginning on day 6 after infection. The plateau of parasites could possibly indicate a small degree of resistance conferred by T lymphocytes in the  $\mu\text{-suppressed}$  mice. To test whether this plateau would be observed in mice made immunodeficient by irradiation, C57Bl/6J mice were irradiated and then infected with T. rhodesiense.

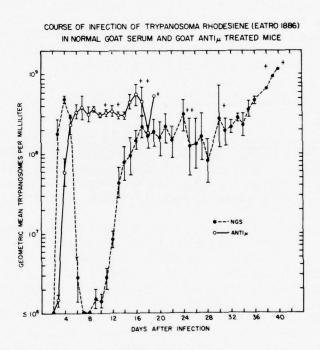


Figure 6 Geometric mean parasitemia in  $\mu$ -suppressed (anti- $\mu$ ) or control (normal goat serum treated, NGS ---) mice after i.p. injection of 106 T. rhodesiense organisms. Each point represents the geometric mean parasitemia of five mice + the standard error.

Control mice or mice subjected to 900 R total body irradiation 24 hrs previously were injected i.p. with 10 T. rhodesiense organisms. The results (Figure 7) again showed a plateau of organisms reached by day 4 after infection in the irradiated mice. Control mice had reduced their parasites to undetectable levels (10 organisms/ml) by day 6. The parasitemia began to increase again in control mice (not shown) by day 10 as in the previous experiment. Even when immunocompetence of mice was reduced by lethal total body irradiation, the logrithmic rate of increase in parasite numbers did not continue beyond day 3 after infection. This suggests that the plateau of parasites was probably not due to an immunologically mediated host response.

c. The effect of immunization on infection of control and  $\mu$  -suppressed mice. Four control or four  $\mu$ -suppressed mice (age matched and of random sex) were injected i.p. with  $10^7 \, \mathrm{irradiated}$  EATRO 1886 organisms seven days before i.p. injection of  $10^3 \, \mathrm{live}$  trypanosomes. The results (Fig. 8) confirmed those resulted shown previously (Fig. 6) and indicated that prior immunization with irradiated organisms had no effect in the  $\mu$  -suppressed mice on the development of parasitemia after challenge with viable organisms. Control mice that had been immunized with irradiated organisms did not develop detectable circulating parasites after challenge with live organisms.

# 2. Studies on the response to challenge of T. rhodesiense immunized animals

a. Antibody production in response to challenge. Mice immunized with 60 KR irradiated cloned trypanosomes of the Wellcome strain were used as donor mice in adoptive spleen transfer system. This transfer system was designed to allow the measurement of a secondary antibody response, by the transferred spleen cells, in the recipient animal.

Spleen cells were taken from donor C57BL/6 mice at various times after primary immunization with 1 x  $10^7$  irradiated Wellcome organisms. These spleen cells were washed, counted, and 50 x  $10^6$  transferred to each syngenic recipient. The recipient mice had received 100 rads of total body gamma radiation immediately prior to the cell transfer. At two hours after receiving spleen cells from either immune or normal donors, recipient mice were given 1 x  $10^3$  irradiated Wellcome organisms i.p. This dose of trypanosome antigen had previously been found to elicit a secondary but not a measurable primary antibody response. Serum antibody titers were measured in the recipient mice at seven days after the cell transfer by a direct agglutination test using live irradiated Wellcome trypanosomes. The results are shown in figure 9.

A low level of antibody was detectable in the recipients of five day immune cells. The antibody produced increased with time and peaked in recipients of 20 day immune spleen cells. Cells taken at longer times

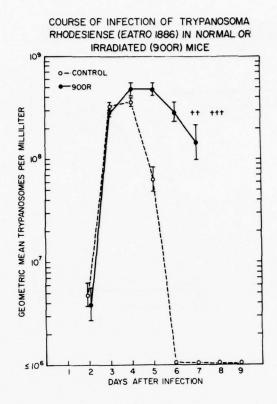
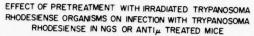


Figure 7. Geometric mean parasitemia in normal (---) or irradiated (900R) mice after i.p. injection of  $10^6$  T. rhodesiense organisms. Each point represents the geometric mean parasitemia of five mice  $\pm$  the standard error. Mice were injected 24 hrs after irradiation.



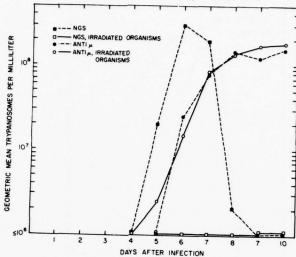


Figure 8. Effect of pretreatment of  $\mu$ -suppressed (anti-u) or control 7 (NGS) mice with 10 irradiated trypanosomes seven days before challenge with 10 live organisms. Geometric mean parasitemias of four mice per group are shown for nonimmunized ( NGS, • anti- $\mu$ ) mice or mice immunized with irradiated trypanosomes (NGS, • anti- $\mu$ )

after primary immunization yielded essentially the same level of antibody. Although not shown here control mice receiving only immune cells and no antigen, produced no detectible antibody. Also mice receiving normal spleen cells plus antigen had no detectable antibody.

b. Resistance to challenge in adoptive spleen cell transfer. Mice having received spleen cells from normal or immunized mice, as described above, were challenged at 24 days with 1 x 10 live Wellcome trypanosomes. The course of resistance to challenge is shown in figure 10 as the percent survival of each group challenged. Only partial protection was seen in mice receiving either five or ten day immune spleen cells, whereas mice receiving 20 day or beyond immune cells showed 100% protection to challenge. No significant resistance to challenge was seen in recipients of immune cells and no antigen.

## c. Lymphocyte subpopulations active in the response to challenge

In order to determine the relative contribution of T and B lymphocytes to long term immunity, nylon wool fractionated spleen cells were used in the adoptive transfer system described in the previous sections.

Spleen cells taken from mice immunized, three to four months previously with 1 x 10 irradiated trypanosomes of the Wellcome strain, were fractionated by the nylon wool technique as previously described. Recipient mice were given 50 x 10 6 unfractionated, T enriched or B enriched spleen cells. As before the cell transfer transfer was followed at two hours by an i.p. injection of 1 x 106 irradiated Wellcome trypanosomes. As shown in figure 11 mice receiving unfractionated or T enriched spleen cells had comparable antibody levels while mice receiving B enriched cells had significantly lower levels of antibody. Animals receiving only immune cells and no secondary dose of antigen had no detectable antibody.

D. Discussion:

The course of infection induced by  $\underline{T}$ . rhodesiense was examined in C57BL/6J mice which had been treated with goat antiserum directed against  $\mu$  heavy chains and which were thus deficient in B lymphocytes. On challenge with the organisms control animals treated with normal goat serum exhibited first a peak and then a spontaneous decline in parasitemias; in contrast, the B cell deficient animals exhibited no such spontaneous remission. Furthermore, B cell deficient mice died earlier than control mice. Immunization with irradiated organisms induced complete resistance to infection in controls but not in the B cell deficient animals.

These findings have demonstrated the necessity of B cell effector mechanisms for resistance to African trypanosomiasis. There was no evidence for any T cell effector mechanism in the absence of antibody. However, the adoptive transfer studies using nylon wool fractionated spleen cells implicate T cells, at least in a helper capacity in the response of immunized mice to challenge.

TIME COURSE OF SECONDARY RESPONSIVENESS IN ADOPTIVE SPLEEN CELL TRANSFER

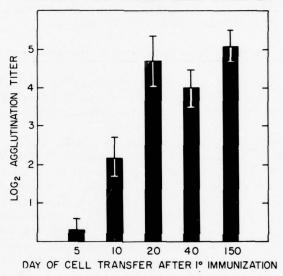


Figure 9.

Time course of secondary responsiveness in adoptive spleen cell transfer. The antibody response to  $1\times10$  irradiated trypanosomes is shown in mice which received 50 x 10  $^6$  spleen cells from donors immunized at various times prior to cell transfer. The bars represent the mean  $\log_2$  agglutination titers  $\pm$  standard error.

III Antibody-Dependent Cell-Mediated Cytotoxicity Against the Circulating Blood Stage of Trypanosome rhodesiense.

- A. Objective: The objective was to design in vitro experiments in order to determine if antibody dependent cellular (ADCC) cytotoxicity against the trypomastigote stage of  $\underline{T}$ . rhodesiense could be mediated by mouse spleen cells.
- B. Description: Resistance to T. rhodesiense has been transferred by immune serum and by B-lymphocytes from immune donors. Trypanosomes coated with specific antibodies lyse when complement is present. However, when trypanosomes are incubated with antibody and subsequently inoculated into mice that are genetically C-5 deficient or that have been depleted of late complement components by prior treatment with cobra venom factor, no infection occurs, indicating a mechanism of resistance to trypanosome infection that appears to be complement independent. Such a mechanism of resistance is suggestive of ADCC against trypanosomes.

We have developed a 3H leucine release technique to test for in vitro cytotoxicity to trypanosomes. The release of previously incorporated H leucine was used to detect cytotoxicity to the trypanosome. In brief, trypanosomes were incubated at 37 C for 2 hrs with H leucine (10 ci/ml) in a mixture of equal volumes of 5% glucose and leucine-free medium 199. Trypanosomes, after three washings were incubated for 2 hrs at 37 C, 5% CO<sub>2</sub> in 400 µl microcentrifuge tubes with normal mouse serum or immuhe mouse serum (five weekly injections of 1 x 10' irradiated trypanosomes per mouse) heated at 56 C for 30 minutes with or without complement (guinea pig serum stored at -70 C). Two hundred and ten  $\mu$ l of a mixture of serum and/or leucocytes and 1 x 105 trypanosomes made up each reaction mixture. Each tests was performed in triplicate. After the two hour incubation microcentrifuge tubes were centrifuged (1000 x G) for four minutes and 50  $\mu$ l of the supernatant was placed on a filter paper disc, dried and counted using a Packard liquid scintillation spectrometer. The total amount of leucine was determined by drying 1 x 10° trypanosomes directly on the paper disc. Distilled water lysis of trypanosomes was considered to induce maximal leucine release. Spontaneous leucine release was estimated after incubation in normal mouse serum. The percentage of trypanosome cytotoxicity was determined by the formula:

Experimental leucine release - spontaneous leucine release x 100 Maximal leucine release - spontaneous leucine release

Statistical significance was determined by using the Student's t test and and the null hypothesis was rejected when values of P were less than 0.05.

C. Progress: In figure 12, the percent cytotoxicity of trypanosomes induced by normal mouse spleen cell plus immune serum or normal

There was no cytotoxicity of trypanosomes spleen cells is shown. when normal cells plus normal serum were used, but in the presence of immune serum a significant increase in cytotoxicity was observed in all (except the 10:1) spleen cell to trypanosome ratios. A 20:1 spleen cell to trypanosome ratios appeared to be the best for future experiments. We next examined the role of immune mouse spleen cells and the role of complement on in vitro cytotoxicity of trypanosomes again represented as the percentage leucine release. As was seen, figure 12 and figure 13 show a significant increase in the percentage of cytotoxicity to trypanosomes when incubated with normal spleen cells and immune serum. Immune spleen cells, however, did not significantly change the percentage of cytotoxicity to trypanosomes incubated with or without immune serum as compared to that observed with normal spleen cells. The addition of complement to the incubation mixtures did not consistently result in increased cytotoxicity under the conditions employed. It thus appears that in vitre cytotoxicity of trypanosomes, as measured in our system, does not occur with immune (sensitized) spleen cells alone even though these same mice have enough antibody in their serum to mediate ADCC. Furthermore, complement does not appear to augument the degree of in vitro ADCC.

D. Discussion:

These experiments have established that antibody dependent cellular cytotoxicity is mediated against T. rhodesiense by mouse spleen cells when the trypanosome is incubated in vitro with an homologous antibody. The addition of complement did not consistently result in increased cytotoxicity. Immune (sensitized) mouse spleen cells did not mediate cytotoxicity without immune serum present and even when in the presence of immune serum did not significantly increase the cytotoxicity above that observed with normal spleen cells and immune serum. Future studies are designed to identify the cell types that mediate the ADCC and to identify the class or subclass of antibody responsible for directing the ADCC.

IV Isolation and purification of protective antigens from T. rhodesiense

- A. Objective: The purpose of this study was to explore an additional method of extract preparation as as a source material for antigens and to provide a methodology for identifying and isolating protective antigens and to provide a methodology for identifying and isolating protective antigens responsible for variant specificity.
- B. Description: The method of Cross for preparing soluble trypanosomal protein extracts was chosen for study because it involved several procedural steps that appeared to provide a stable source material from which additional isolation procedures could be carried out. Analyses of the crude extracts were accomplished by the use of analytical isoelectric focusing in thin layer acrylamide gels and the separated protein components were identified by Coomassie brilliant blue (R250) staining. Monitoring of the antigens was done either by immunoelectrophoresis or by immunodiffusion in agarose using sera obtained by immunization with irradiated live organisms, soluble extracts and antigen antibody complexes developed by immunodiffusion in agarose.

C. Progress: Four major peaks were obtained by DEAE-cellulose chromatography using Cross' method for preparing antigen extracts. The breakthrough peak designed as Cross' antigen, contained the bulk of the antigen activity although occassional reactivity was observed in Peak III with some antisera. Comparison of Cross' antigen with KCL extracts and 21 hour saline extracts indicated that it was comparable in activity to KCL extracts and both were approximately twice as reactive as saline extracts. Isoelectrofocusing on thin layer acrylamide gels showed a number of major components with pI's ranging from 8.2-9.3. Immunodiffusion of the isoelectrofocused bands in agarose with antisera derived from the same organisms produced precipitin band corresponding to a protein with a pI of 8.8 for peak three organisms (T. rhodesiense Wellcome strain, CP3 B5 ) and a pI of 9.1 for Peak two organisms (T. rhodesiense, Wellcome strain, CP, A,). Antisera obtained from animals injected with the precipitin antigen - antibody complex protected those mice challenged with the same variant. No protection was obtained when antisera from a different variant was used.

#### D. Discussion:

The preparation of soluble extracts of  $\underline{\mathbf{T}}$ , rhodesiense by the method of Cross provided a suitable source of protective antigens. Evidence was provided to show that a protein identified by immunoassay as a precipitin band was also a protective antigen in that antisera developed against the purified antigen passively protected mice and was variant specific.

- V. Immediate hypersensitivity reactions in trypanosomiasis:
- A. Objective: A previous annual report demonstrated that basophils from rabbits infected with T. rhodesiense when incubated with specific antigen, degranulated and released their stores of histamine. These earlier results also showed that the amount of platelet activating factor (PAF), as demonstrated by histamine release from normal platelets, was limited. These studies were again initiated to determine if a true immediate hypersensitivity reaction involving basophils, eosinophils and platelets, with the subsequent release of pharmacologically active mediators, occurs during trypanosomiasis. The first objective of this study was to monitor the hematological changes that occur during T. rhodesiense infection in rabbits. Emphasis was placed on those cells involved in immediate hypersensitivity reactions, namely basophils, eosinophils and platelets.
- B. Description: Three rabbits were injected intravenously with 10<sup>6</sup> T. rhodesiense Wellcome strain. Each rabbit was then bled approximately every other day for 70 days after infection. Three control rabbits not injected with organisms, were treated exactly the same as the infected animals. Direct counts of basophils, eosinophils and platelets were done on each bleeding from each rabbit. For ease of comparison, the results of the three infected and three control rabbits were averaged. It should be noted that individual rabbits generally showed the same pattern changes as the averages.

# TIME COURSE OF RESISTANCE TO LIVE CHALLENGE IN ADOPTIVE SPLEEN CELL TRANSFER

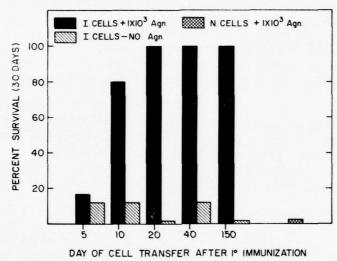


Figure 10.

Resistance to live challenge in the adoptive secondary spleen cell transfer. The percent 30 day survival after live challenge is shown for mice having received 50 x  $10^9$  spleen cells from normal or immunized donors and 1 x  $10^9$  irradiated trypanosomes as a second dose of antigen.

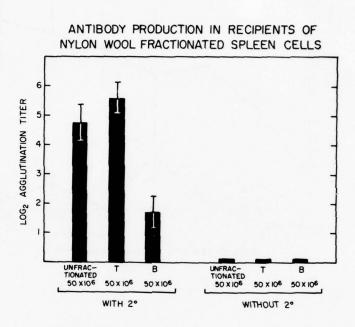


Figure 11. Antibody production in recipients of nylon wool fractionated spleen cells. The mean log, antibody titer  $\pm$  standard error is shown for mice receiving the indicated spleen cell population either with or without a secondary dose of  $1 \times 10^6$  irradiated trypanosomes.

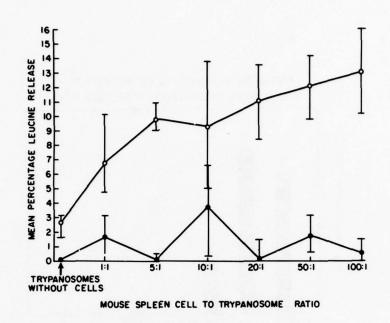


Figure 12

The percentage cytotoxicity (represented as mean percentage leucine release) of trypanosome in normal mouse serum o o and in hyperimmune mouse serum when incubated with ratios of normal mouse spleen cells to the trypanosomes of from 1:1 to 100:1. The cytotoxicity of water lysis of the trypanosomes for these experiments was 13.7% of the total leucine release by drying trypanosomes on filter paper disc. Vertical bars represent the range. The cytotoxicity observed with immune sera and normal spleen cells was statistically significant in all cell to trypanosome ratios except the 10:1 ratio which was significant at the 0.5 level.

TABLE I

Percent Changes in Basophils, Eosinophils and Platelets that
Occur during Trypanosomiasis

Days Post	CONT	ROL RABBIT	'S	INFECTED RABBITS			
Infection	Basophils	Eosinophils	Platelets	Basophils		Platelets	
2	+16.5	+26.7	+33.3	+4.7	-55.0	-24.8	
4	-21.7	+56.9	+103.7	+1.3	-81.0	-49.4	
7	-27.6	+44.1	+85.0	-1.8	68.7	+12.9	
9	-24.2	-38.1	+122.2	+28.7	-89.9	-40.4	
11	-33.4	-20.3	+152.3	-10.9	-94.9	-31.3	
16	+2.4	-33.6	+9.4	-30.4	-87.7	-41.2	
18	0	-8.6	+53.1	-25.7	-85.0	-24.9	
21	+2.6	-5.4	+71.3	-18.4	-77.3	-26.5	
23	+3.2	+10.1	+36.9	+5.7	-64.6	-13.4	
25	+29.2	+11.6	+81.1	-3.5	-77.2	+30.6	
28	-15.4	+3.6	+41.8	-22.2	-89.6	+22.4	
30	+1.3	+19.1	+28.1	+25.6	-84.2	+33.6	
32	-21.8	+5.6	+22.4	+45.5	-72.9	-9.3	
35	-35.0	+7.8	+10.6	-11.6	-76.3	-31.1	
38	-11.4	-55.2	+31.5	-40.0	-85.9	-9.9	
42	-14.4	-18.4	+69.7	+33.8	-79.7	+38.6	
45	-15.6	+12.4	+78.3	+16.1	-59.7	+131.4	
49	+10.0	+3.7	+15.8	+22.3	-43.9	+32.4	
52	-28.4	+2.4	+68.7	+56.7	-22.3	+52.3	
56	+4.3	+8.6	+33.0	+16.1	+39.2	+32.8	
59	-10.9	+23.6	+24.0	+43.4	+39.4	+58.5	
64	+10.2	+1.4	+33.1	+52.3	-6.3	+90.8	
67	-16.2	-13.9	+6.5	+82.9	+25.9	+39.5	
71	+23.2	+14.7	+92.2	+66.9	-19.1	+9.9	

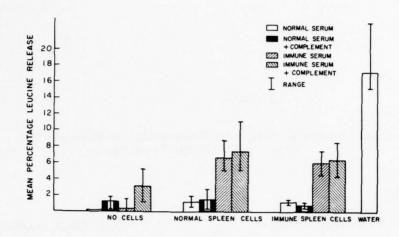


Figure 13

The percentage cytotoxicity (represented as mean percentage leucine release) of trypanosomes incubated in normal mouse serum with or without complement and in hyperimmune mouse serum with or without complement. Water lysed trypanosome represented the maximal leucine release. Vertical bars represent the range over six experiments. Significantly more leucine was released from trypanosomes incubated in immune serum and normal spleen cells as compared with serum and no cells (P 0.05%). Also immune spleen cells mediated a significant leucine released with immune serum (P 0.05%) when compared with immune serum and no cells. The ratio of mouse spleen cells to trypanosomes was 20:1.

C. Progress: The results of the hematological changes that occurred in infected rabbits during trypanosomiasis are listed in Table I. Although the data show considerable variation, there are a number of patterns that are apparent. First, the most striking change occurred in the percentage of eosinophils in the infected rabbits. There was a substantial and persistant decrease in the number of eosinophils compared to the noninfected rabbits throughout most of the infection. Second the percent fluctuation in the number of basophils from non-infected and infected groups were not different early in the infection, however, there was a definite increase in the number of basophils in the infected rabbits late in the infection. Third, there was a decrease in the number of platelets in the infected animals in the early part of the infection and in the latter part of the infection there was an increase in the number of platelets. The non-infected rabbits consistently demonstrated an increase in the number of platelets.

#### D. Discussion:

The most surprising results of this study was the substantial decrease in the number of eosinophils and the lack of persistent thrombocytopenia the infected rabbits. It is not clear at this time if the eosinopenia that develops in these rabbits is related to trypanosomiasis. There are many conditions -stress, chronic inflammation and hormone changes-that are associated with eosinopenia.

- VI. Isolation and Characterization of a new serodeme of <u>Trypanosoma rhodesiense</u>
- A. Objective This work was designed to develop techniques and reagents for further epidemiological, entomological and immunological studies of T. rhodesiense.
- B. Description: Much of the research work in trypanosomiasis has involved the use of laboratory strains of T. rhodesiense with somewhat nebulous histories. These strains have been maintained in the laboratory, often by serial passage for several years since their isolation from a naturally infected host. Many workers have reported changes in several characteristics of particular trypanosome isolates after laboratory passage, i.e., infectivity chronicity, morphology and major antigenic type. Based on these findings, it was suggested that established laboratory strains would not be optimum for further immunization and transmission studies. Also these strains would have limited value for epidemiological studies in currently endemic areas. Therefore, an isolate of T. rhodesiense was obtained from an infected patient in the Lambwe Valley of Kenya to provide the starting material for further studies. This new isolate was handled in a manner, that based on current knowledge would help preserve it's original characteristics. of laboratory passages was limited through the use of frozen stabilates as a source of infectious material rather than constant passage in animals. The passage required were performed in lethally irradiated animals to preclude any immune response to the parasites. trypanosomes were cloned to provide relatively homogenous organisms for study.

Antisera was prepared to surface antigens of individual clones for use in an indirect flourescent antibody assay. This assay provided a sensitive techniques for the detection of organisms of a known antigenic type.

#### C. Progress:

1. Development of trypanosome clones. The original isolate of trypanosomes used in these studies was obtained from a patient with rhodesign type trypanosomiasis in the Lambwe Valley of Kenya. Blood from a rat infected with organism from this patient was used to form the initial stabilate. This frozen material was transferred to WRAIR where it was cloned from mouse passage. Cloning was achieved through the injection of mice with single trypanosomes obtained by direct microscopic selection. Serial whole blood passage was performed at four day intervals using 900R irradiated mice until the parasitemia was patent. At this point stabilates were prepared and stored at -70 C for future use as infectious material. In order to obtain trypanosomes of many antigenic types and also to investigate the course of infection with this parasite in laboratory animals, a chronic infection was started in mice using one of the above clones.

Nine C57 BL/6J mice were infected each with lxl0 trypanosomes given intraperitonally. A chronic infection resulted, lasting for 120-150 days, and ending in the death of the animals. As shown in figure 14 the infection was clearly relapsing, with parasitemias ranging from 1x10 per ml down to undetectible levels. During peaks of parasitemia, blood was taken by tail bleedings for the preparation of stabilates. These cryopreserved organism were then cloned by the method described earlier. Figure 15 is a flow diagram of the development of 20 clones in this manner. Of the nine mice inoculated with trypanosomes only eight developed detectable parasitemias. Stabilate populations were derived from these mice at the times indicated e.g., ld 12 represents a stabilate prepared with blood taken from mouse number one on the twelfth day of infection. Clones derived from these populations are represented by capital letters. The subscripts indicate the number of serial four day passes that were performed to yield a given stabilate. Twenty clones were obtained and preserved in this manner.

## 2. Initial characterization of the trypanosome clones

For a preliminary determination of the antigenic uniqueness of each of the clones relative to the others, an agglutination assay was used. Mouse antisera, obtained through immunization with irradiated organism of each clone, were assayed for their ability to agglutinate organisms of each of the clones. The results of this assay are shown in figure 16. Ten of the clones were found to be antigenically unique, i.e., these clones were agglutinated only by their homologous antiserum. Clone 1d 12 agglutinated spontaneously and therefore could not be assayed by this method. The other nine clones showed some degree of cross reactivity among one or more clones. Generally, this was a two-way cross reaction indicating complete antigenic homology between the particular clones.

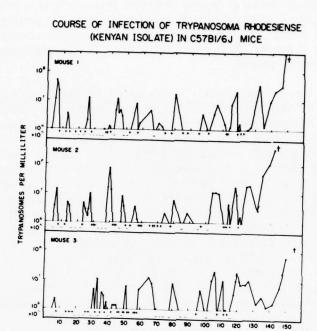


Figure 14.

Parasitemias, expressed as trypanosomes per milliliter, in C57Bl/6 mice during a chronic infection with a new isolate of Trypanosoma rhodesiense. At the times during infection when parasitemias were too low to be accurately counted, the presence or absence of parasites as determined by wet mount is indicated

A total of 13 different (noncross reacting) antigenic types of try-panosomes were found out of the 20 clones obtained from the chronic mouse infection. Most of these 13 were derived from organisms occuring in the second peak of parasites. As many as for antigenically different clones were obtained from the second peak in a single mouse. These observations indicate a great heterogeneity in the organisms comprising a single parasitemia peak.

3. Development of variant specific fluorescent antibody assay.

A modification of the indirect fluorescent antibody assay system described by Van Mervenne was used. Clone specific antisera were prepared by immunizing rabbits with acid-saline preparations of trypanosome surface coat antigens. Pools of sera from these rabbits were used in the fluorescent assay. In this assay, dried blood smears obtained from heavily infected mice were used as the trypanosome antigen. Various dilutions of rabbit sera were reacted with these smears, followed by goat anti-rabbit serum conjugated with fluorescein isothiocyanate. Useful titers of the rabbit sera were determined by reaction with homologous and heterologus trypanosome clones. In general, the homologous titer ranged from 1:128 to 1:2048 while the heterologous reactivity remained at 1:8 or 1:16. For use in an assay to detect trypanosomes of a known antigenic type, the titrated rabbit sera were used at one two fold dilution lower than that which gave a strong positive reaction.

# 4. Critical characterization of the trypanosome clones.

The fluorescent assay described above was used to evaluate the homogeniety of each of the trypanosome clones. Blood smears were prepared from mice infected with individual clones, four days previously. These smears were reacted with the clone specific rabbit antisera and fluorescein conjugate as described above. By this procedure it was possible to determine that each of the clones contained from 0 to 200 nonreactive trypanosomes per 10,000 counted. These organism not reacting with the homologous antisera were presumably variants that arose spontaneously after cloning.

D. Discussion: The reagents and assay system described in this work provide methodology to study variant populations of a recently isolated strain of the human pathogen T. rhodesiense in nature as well as in experimental animal models. The high parasitemia and chronicity of the infection in mice with this serodeme of T. rhodesiense make possible the direct typing of the occurrence of variants during infection that has not been possible with other strains of organisms.

# DEVELOPMENT OF CLONES OF TRYPANOSOMA RHODESIENSE (KENYAN ISOLATE)

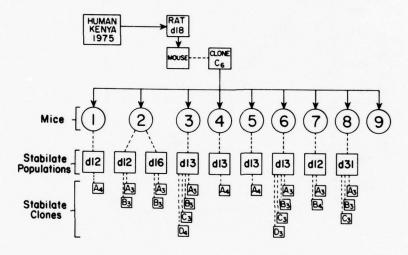


Figure 15. Development of <u>Trypanosoma rhodesiense</u> clones. Mice I through 9 were infected with clones organisms. The stabilate populations were obtained from these populations. Subscripts on the capital letters indicate the number of passes from cloning.

## CLONE SPECIFIC AGGLUTINATION WITH MOUSE ANTISERUM AGAINST IRRADIATED TRYPANOSOMES

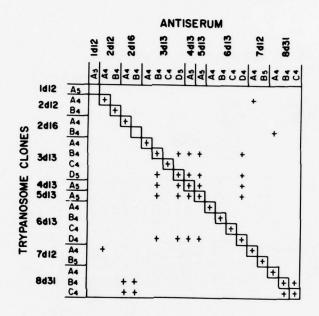


Figure 16.

Clone specific agglutination with mouse antiserum against irradiated trypanosomes. Positive agglutination reaction at a 1:5 dilution of antiserum are indicated.

Projec3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 202 Antigenic composition of trypanosomes

#### Literature Cited.

### Publications:

- 1. Campbell, G.H., and Phillips, S.M.: Adoptive transfer of variant specific resistance to Trypanosoma rhodesiense with B lymphocytes and serum. Infection and Immunity, 1144-1150, 1976.
- 2. Campbell, G.H., Weinbaum, F.I., and Esser, K.M.: Trypanosoma rhodesiense infection in B-cell deficient mice. Infection and Immunity, Nov. 1976 (In press).
- 3. Campbell, G.H., Esser, K.M., and Diggs, C.L.: Abstracts of the annual meeting of the American Society for Microbiology, 98, 1977.

RESEARCI	H AND TECHNOLOGY	WORK UNIT S	UMMARY	DAOC 6440 77 10 01			DD-DR&E(AR)636		
1. DATE PREV SUM'RY 76 10 01	D. Change	S. SUMMARY SCTY	A. WORK SECURITY	7. REGR		NL	ON SPECIFIC	A ACCESS	A WORK UNIT
IO. NO./CODES:*	PROGRAM ELEMENT	PROJECT	NUMBER	TASK	REA NUMBER			T NUMBER	
- PRIMARY	61101A	3A16110	1A91C		00	206			
b. CONTRIBUTING									
c. CONTRIBUTING									
	Influencing	Secretory	States						
	ECHNOLOGICAL AREAS								
003800 Life	Support		O Stress P						
13. START DATE		14. ESTIMATED COM	PLETION DATE	IS FUNI	HING AGENCY		16. PERFOR	MANCE MET	HOD
75 07		CONT		DA		1		In-Ho	
17. CONTRACT/GRANT				10. RES	DURCES ESTIMAT	E & PROFE	SIONAL MAN Y	AS & FUN	DS (In thousands)
A DATES/EFFECTIVE	NA NA	EXPIRATION:			77		2		75
L NUMBER:		& AMOUNT:		PISCAL	CURRENT	-		-	
& KIND OF AWARD:					78		1		84
19. RESPONSIBLE DOD	ORGANIZATION	f. CUM. AMT.		20. PER	ORMING ORGANI	HOITAS			
NAME: Walter Reed Army Institute of Research  ADDRESS: Washington, D.C. 20012  NAME: Walter Reed Army Institute of Res Division of Surgery ADDRESS: Washington, D.C. 20012					Research				
TELEPHONE: (20) 21. GENERAL USE Foreign int	mund, COL, G. 2) 576-3551 elligence not			PRINCIPAL INVESTIGATOR (Pumirsh SEAR II U.S. Academic (material)  NAME: Trout, MAJ, Hugh H. III  TELEPHONE: (202) 576-3284  SOCIAL SECURITY ACCOUNT NUMBER:  ASSOCIATE INVESTIGATORS  NAME: Harmon, MAJ, John W.  NAME:					
	cidity; (U)		U) Trauma	; (U	) Gastr	ic Acid			
secretion as is relevant in up to 10 of these ul and removal 24 (U) Gas pump system while gastr loss is being 25 (U) 76 acid secret antrectomy Studies of to histamin gastrin or acid secret proximal ga acidity, was sound to vagotomy re-	cidity: (U) TIVE.* 24 APPROACH. 28. technical obj nd of duodenal to military s per cent of i cerations so i from the stom tric acid secr to instill gr ic acid is col ng measured us 10-77 09 The ion has been a slightly incre gastric secret e was about 2X the full 17 am ion in respons stric vagotomy s carried out o greatly redu duced acid sec	acid remo surgery bec njured pat ncreasing ach is of retion is b aded doses lected and sing a reci effect of assessed in asses acid dion in mon as great tino acid g to these (PGV), wh in dogs. ace gastric retion abo	val. Blee ause it oc ients. Ga our unders potential eing measu of the se the acidi rculating antral den dogs. An secretion keys revea as that in astrin mol secretago ich is a n Of the 2 c acidity but 15 per	ding curs strict and in it red i creta ty me pH stervat tral in re led t respecule gues ew su ompon y abo cent.	from streas a sign acid ple ng of the ary impore n dogs ar gogues, hasured by at systemion and denervat: sponse to hat maxim onse to e. In maris equal rgical prents of hut 70 per note to	ess ulconificant ays a re control ctance. add monke distamin titrai n with a chen sub con has o histam and acid either a physic cocedure GV, les r cent v	eration t clinic t cl	of the all prohe pat id sec gan i etraga urette antre ct whi tetra ion in trin, p maxima testimuce ga vature ophage	e stomach oblem hogenesis eretion unfusion estrin al acid ectomy on the egastrin. I response enta- el gastric e vagotomy al
1 July 76 -	al report see 30 Sept 77.		d Army Ins	titut	e of Rese	earch Ar	nnual Pr	ogress	Report

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 206 Factors influencing secretory states

Investigators

Principal: Hugh H. Trout, III, MC Associate: John W. Harmon, MAJ, MC

### I. Antrectomy in Dogs

- A. Background and Statement of the Problem. Resection of the antrum and duodenal bulb in man results in a decrease in maximal histamine stimulated gastric acid secretion. In marked contrast to these findings in man, it has been shown in dogs and cats with vagally innervated gastric pouches (Pavlov pouches) that antrectomy results in an increase in gastric acid secretion in response to either of the stimulants histamine or pentagastrin (1,2,3). The present study was undertaken to determine if these differences were species related or were due to the different types of preparation (essentially a gastric fistula in man as opposed to isolated gastric pouches in the experimental animal). In addition the investigations were modified in an attempt to explore possible causes for these differences.
- B. Experimental Approach. Dogs were prepared with gastric fistulas and dose response data were collected using histamine and tetragastrin as stimulants and acid output as response. The vagal nerves to the antrum of the stomach were divided and the studies were repeated.
- C. Results and Discussion. Division of the vagal fibers to the antrum had little effect on acid output in response to either histamine or tetragastrin. Antrectomy increased acid response to both stimulants slightly. These results indicate that the earlier work with pouches is a true reflection of the acid secreting response following antrectomy in the dog or cat. It demonstrates that these differences are species related and are not due to alterations created by the construction of fundic pouches. The results showing no significant change after division of the vagal nerves to the antrum show that the increased acid secretion seen after antrectomy are not due to any interference with vagal innervation of the stomach or small bowel when the antrectomy is performed. Follow-up studies of these results are underway to evaluate the role of gastrin in the hyper-secretion after antrectomy. Both serum gastrin and gastric mucosa gastrin are being measured before antrectomy and then 1 and 6 months after antrectomy.

# II. Histamine and Gastrin in Monkeys

- A. Background and Statement of the Problem. There is a continuing controversey over substances ultimately responsible for gastric acid secretion. Gastrin, histamine and acetylcholine have each been proposed as being the primary stimulant. In cat, dog and human, gastrin and its analogs elicit the same maximal gastric acid output as does histamine. The monkey, however, has not yet been carefully studied (4). Since the monkey is phylogenetically closely related to the human, a more thorough comprehension of the factors regulating gastric acid secretion in the monkey may prove to be of benefit in better understanding human gastric secretory mechanisms.
- B. Experimental Approach. Rhesus monkeys were prepared with gastric fistulas; dose-response data in conscious non-sedated animals were collected using histamine, tetragastrin, pentagastrin, and gastrin as stimulants and acid output as response. Other studies were performed using histamine and tetragastrin in combination with a continuous infusion of carbachol, and acetylcholine analog.
- C. Results and Discussion. Histamine elicited a peak acid output response which was double that achieved by gastrin or any of its analogs. The addition of carbachol did not significantly alter the acid response to either histamine or tetragastrin. The meaning of these results is unclear but suggests that there are serious gaps in our understanding of the regulatory mechanisms of gastric acid secretion at least as they apply to the Rhesus monkey. Follow up studies of this phenomenon are being carried out. Background infusion of how doses of either gastrin or histamine are being made while a full range of the other secretagogue is infused to see if maximal secretion of these combinations are equivalent. The results of our acid testing to date are shown in the table below:

N	HIST dose (ug/kg-		80	320
	TG dose (ug/kg-hr	) 4	16	64
7	HIST	316+46	602+68	731+140
7	TG	89+24	204+41	289+28
3	HIST	291+46	566+74	653+134
3	HIST + carbachol	314+70	689+154	765+177
3	TG	171+48	288+53	232+54
3	TF + carbachol	223+39	338+44	310+53

### III. Partial Vagotomy in Dogs

- A. Background and Statement of the Problem. Highly selective vagotomy which consists of dividing the vagal nerves to the acid secretion portion of the stomach is an operation which is becoming increasingly popular. The advantages of this operation are that the gastrointestinal tract does not need to be opened and there are fewer postoperative side effects. Essentially the operation consists of two parts first the division of vagal fibers along the lesser curvature of the stomach which innervate the acid secreting portion of the stomach and second the division of fibers around the esophagus. This study was designed to determine what the relative effect of each of these two components of the operation have in acid secretion.
- B. Experimental Approach. Two groups of 4 dogs were prepared with gastric fistulas. Control studies were undertaken testing with histamine, tetragastrin and 2-deoxy-d-glucose. One group then had the vagal fibers to the less curvature divided and the other group had the fibers around the esophagus transected. The dogs were then retested and then had the remainder of their fibers divided thus completing a highly selective vagotomy in each dog. Final repeat testing was then carried out.
- C. Results and Discussion. The results of this gastric acid secretory testing in response to graded doses of 2DG are shown in the table below. Acid secretion is in uEq/10 min.

Control EV	5	428 <u>+</u> 185 312+162	2917 <u>+</u> 892 2524+877	4435+372 3511+749
Control	5	221 <u>+</u> 86 8+4*	2841 <u>+</u> 514 1153+469*	4758 <u>+</u> 813 1348 <u>+</u> 338**
Control	6	303+64	2810+292	3760+234
EV+LCV	6	51 <u>+</u> 48*	555+271***	640+226***

The reduction was greatest after EV+LCV, less marked after LCV alone and not statistically different for EV alone: p by t test 40.05\*, 40.01\*\*, 40.001\*\*.

#### IV. Duodenal Mucosa

A. Background and Statement of the Problem. The duodenal mucosa has recently been shown to have a significant capacity to dispose of acid without contribution from the pancreas of liver. A defect in duodenal disposal of acid could result in increased gastric acidity by slowing the gastric emptying of acid. This mechanism could be important in the pathogenesis of gastric as well as duodenal ulceration.

- B. Experimental Approaches. An experimental preparation has been developed using a duodenal pouch in a chronic dog with 2 Gregory cannulas and a recirculating system through a reservoir with a pH stat titrator system. This allows minute to minute measuring of duodenal acid loss at varying rates of perfusion and at varying acid concentrations. The questions of the relative influence of rate and concentration, the role of bicarbonate in the system as determined by the influence of acetazolamide, the effects of aspirin and bile salts and the effects of cyanide are all being studied.
- C. Results and Discussion. Results show that the rate of acid loss is dependent on both the concentration of acid and the rate of perfusion. Diamox inhibits acid loss and this varies at different acid concentrations. Cyanide was found to increase the rate at which acid disappears from the duodenal lumen. In the table below the rate of acid loss from the lumen is shown at pH3 in column A and at pH3 with the addition of 10mM sodium cyanide in the column labelled A+C. The mucosal potential difference (PD) and the duodenal pO2 (PdO2) and the duodenal pCO2 (PdCO2) were affected by cyanide.

	<u>A</u>	A+C	
AH+(uEq/10 min)	-111+8	-251+33	0.01
PD (mV)	-9+1	-8+1	N.S.
P <sub>d</sub> CO <sub>2</sub> (mm Hg)	45+7	40+6	N.S.
$P_dO_2$ (mm Hg)	121+6	126+5	N.S.

In another series of experiments evaluating duodenal acid loss  $pCO_2$  was measured in the duodenal lumen while acid was being removed. The  $pCO_2$  measurements were taken with a mass spectrometer in collaboration with MAJ MONTY WOODS. A linear correlation between the rate of acid loss and  $pCO_2$  is evident in the table below:

	pH	9	7	4-6	2-3	1.3-1.7
N	_	$\overline{2}$	2	7	7	4
JH+ - uEq/10 min		-60+0	7+5	114+13	307+34	729+59
∆pCO <sub>2</sub> mmHg		- 5 <del>+</del> 7	-1 <del>+</del> 8	11+3	32+6	64+16

For the 22 experiments  $\Delta pCO_2$  correlated positively with acid loss (JH+) with p <.001 according to this equation:  $\Delta pCO_2=0.08$  JH+ + 3.27. From these experiments we conclude that isolated canine duodenal mucosa has an independent mechanism to dispose of acid by bicarbonate neutralization. Bicarbonate neutralization would produce  $CO_2$  according to the formula  $HCO_3$  Na + HC1  $\nearrow$   $H_2O$  + NaC1 +  $CO_2$ .

### V. Estrogen-like Effects of Cannabinoids

A. Background and Statement of the Problem. The possibility that marijuana could have estrogen-like effects was raised by our initial report of gynecomastia in chronic marijuana users (6) and by a subsequent

report of an animal model in which 9 tetrahydrocannabinol (THC) caused breast stimulation in rats. Marijuana may reduce serum testosterone levels and reduce sperm counts in humans. Marijuana increases uterine weight in rats.

In work we have performed previously at WRAIR THC was found to decrease testicular weight as well as serum and testicular testosterone in rats (9). We are now studying how these effects are produced.

- B. Experimental Approach. In collaboration with DR. ROBERT SHOEMAKER at the AFIP a system for measuring estrogen receptors in tissue cytosols has been developed. The goal of our project has been to determine whether or not THC binds to estrogen receptors in which case they could be presumed to be perceived as estrogen by target tissues.
- C. Results and Discussion. The dextran coated charcoal method (Mester et al., Biochem. J. 120:831, 1970) demonstrated high affinity binding of <sup>3</sup>H-delta-9-THC and its primary metabolite, 11-OH-THC, in uterine and mammary cytosol of immature rats. This binding was strongly inhibited by pretreatment of the cytosol with estradiol, indicating competition for the same binding site(s). The specificity of the binding was further defined by comparing THC binding in estrogen target tissues with binding in non-target tissues and in serum. This binding was found to follow the same hierarchy as binding of <sup>3</sup>H-17 -estradiol: uterus-breast-serum-muscle-kidney. Surcrose density gradient centrifugation of bound THC revealed sedimentation characteristics identical to those of the 4-5 S component of the estrogen receptor complex. While the specifics of the interaction of THC with cellular and extracellular proteins remains to be elucidated, the results of this initial study support binding of THC to estrogen receptors as a mechanism for the estrogenic action of marijuana.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 206 Factors influencing secretory states

## Literature cited.

Publications:

- 1. Trout, HH III, Elashoff, J, Harmon, JW: Gastric acid secretion in gastric fistula dogs after antral denervation and antrectomy. Gastroenterology 73:492-494, 1977.
- 2. Trout, HH III, Carmer, JW, Harmon, JW: Gastric acid secretion in gastric fistual dogs following antral denervation and antrectomy. Gastroenterology 70:944, 1976.
- 3. Trout, HH III, Lewis, CD, Harmon, JW: Gastrin and its analogs are not strong stimulants for gastric acid secretion in the Rhesus monkey. Gastroenterology 70:1141, 1977.
- 4. Harmon, JW, Trout, HH III, Gurll, NJ, Carmer, JW: Effect of proximal gastric vagotomy on gastric emptying of semisolid food slurry. Gastroenterology 70:895, 1976.
- 5. Gurll, NJ, Harmon, JW, Callahan, W: The effect of acid load and acetazolamide on acid loss in distal duodenal pouches. Surgical Forum 27:440-442, 1976.
- 6. Gurll, NJ, Harmon, JW: Canine duodenal mucosal neutralization of acid. The Physiologist 19:213, 1976.
- 7. Harmon, JW, Locke, D, Aliapoulis, MA, MacIndoe, JH: Interference with testicular development by ▲9 tetrahydrocannabinol. Surgical Forum 27:350-352, 1976.
- Shoemaker, RH, Harmon, JW: Suggested mechanisms for the demasculinizing effect of marijuana. Fed. Proc. 36:395, 1977.

### Literature Cited.

### References:

- 1. Bromme, A., and Olbe, L.: Studies on the mechanism of the antrectomy induced suppression of the maximal acid response to histamine in duodemal ulcer patients. Scand. J. Gastroenterology 4:281, 1969.
- 2. Anderson, S., and Grossman, M.: Effect of antrectomy on gastric secretion of acid and pepsin in response to histamine and gastrin in dogs. Gastroenterology 49:246, 1965.
- Dinbar, A., Trout, H., and Grossman, M.I.: Effect of antrectomy on acid secretion from Pavlov pouches in cat. Proc. Soc. Exp. Biol. and Med. 137:393, 1971.
- 4. Rosato, E.F., Mayer, L.H., Arenschield, S., Rosato, F.E., and Brooks, F.P.: Acid secretory responses to histamine and pentagastrin in conscious monkeys. Am. J. Digest, Dis. 19:111, 1974.
- 5. Rosato, E.F., Shumate, G.R., Pollock, T.W., Adair, L., Rosato, F.E. and Brooks, F.P.: Influence of antrectomy on gastrin release and gastric secretion in conscious monkeys. Surgery 77:817, 1975.
- 6. Harmon, J.W., and Aliapoulios, M.A.: Gynecomastia in marijuana users. N. Eng. J. Med. 287:936, 1972.
- 7. Harmon, J.W., and Woods, M.: Canine duodenal mucosal has an independent mechanism for bicarbonate neutralization of acid. Surg. Forum 1977 (accepted).

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY			1. AGENCY ACCESSION 2. DATE OF SUMMARY			MMARY			
				DA UC 0441 // 09 30				R&E(AR)636	
3. DATE PREV SUM'RY	4. KIND OF SUMMARY		WORK SECURITY				CONTRACT	OR ACCESS	LEVEL OF SUR
76 10 01 No. NO./CODES:*	H. Term	PROJECT N	U -		A NUMBER	NL	₩ ves		
a PRIMARY		3A161101A91			00	207	WORK U	NIT NUMBER	
b. CONTRIBUTING	61101A	SAIGIIUIA9I	<u> </u>	_	00	207			
c. CONTRIBUTING									
11. TITLE (Procedo with	Security Classification Code	,•							
(II) Model S	Systems for A	ntiparasitic	Drugs						
002600									
15. START DATE		14. ESTIMATED COMPL	ETION DATE	IL FUNC	HIG AGENCY		IS. PERFO	RMANCE MET	нов
75 07		77 09		DA			C.	In-Hou:	
A DATES/EFFECTIVE:	NA	EXPIRATION:		IO. RES	PRECEDING	E & PROFES	SIONAL MAN	YRS & FUN	DE (In thousands)
h number:*	MA	EXPINATION:		FISCAL	7.6		7.5		113
G TYPE:		4 AMOUNT:		YEAR	ZURNENY	+	1.75		113
& KIND OF AWARD:		f. CUM. AMT.			77		2.30		317
19. RESPONSIBLE DOD	DRGANIZATION			20. PER	ORMING ORGANI		1		T
MAME: Walter	Reed Army In	stitute of I	Research	-	Walter	Reed Ar	ny Inst	itute	of
Walter				1		h, Div.	The same of the same of		
ADDRESS: Wash	ington, DC 2	0012		ADDRES	h. <b>•</b>				
PRINCIPAL INVESTIGATOR (Pumirà MAN II U.S. Academic Institution)									
RESPONSIBLE INDIVIDU	APMUND, COL G			Davidson, David E. LTC					
2	02-576-3551				HONE: SECURITY ACCO		-2292		
TELEPHONE: 2	02 370 3332			-	TE INVESTIGATO				
				HAME:	and the same of th	aux, P.	S.		
Foreign in	telligence no	t considere	i	NAME:		rling,		CPT	
22. KEYWORDS (Procedo	BACH with Society Classifi	catton Code) (U)	Leishmania	sis;	(U) Schi	stosomi	asis;	(U) Dru	g
Developmen	t; (U) Anti-p	arasitic; (	J) Animal	Mode	ls; (U) T	rypanos	omiasis	3	
The second second second	IVE. 24 APPROACH, 25.								
	o develop new								
developmen	t of drugs to	prevent and	treat pa	irasi	tic disea	ses in	militai	ry pers	onnel.
24. (U) D	evelopment of	animal mod	al test si	ctom	c capable	of ran	idly o	roonin	a large
	chemical com						Idly St	.i eeniin	g rarge
nambers of	Chemical con	pounds for	enerr uner	par	asitic ac	civicy.			
25. (U) 7	6 10 - 77 09	Studies to	determine	the	effectiv	eness o	f IA-3	N-0xid	e and
IA-4 N-oxi	de against S.	mansoni in	fection in	rhe	sus monke	ys were	comple	eted.	Both
drugs cure	d infections	when admini	stered by	oral	(P0) or	intramu	scular	(IM) r	outes.
	he best of a								
	ctive as gluc								tromys
albicaudatus was shown to be an excellent model for L. tropica infection.									
In vitro	In vitro screening of antitrypanosomal drugs has been initiated, and a good degree								
of correlation with in vivo activity has been observed.									
or corrected with in vivo activity has been observed.									
Suitable m	odels to test	antileishm	anial. ant	isch	istosomal	and an	titrvpa	anosoma	l drugs
	een establish								
	nue under 3 n								
Walter Ree	d Army Instit	ute of Rese	arch Annua	al Pr	ogress Re	port 1	Jul 76	- 30 J	ul 77.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 207 Model Systems for Antiparasitic Drugs

Investigators

Principal: LTC David E. Davidson, Jr., VC

LTC Peter S. Loizeaux, VC
MAJ Robert E. Desjardins, MC
MAJ Larry D. Hendricks, MSC

Marie M. Grenan

Associate: MAJ Willis Reid, MSC

Schistosomiasis, Leishmaniasis, and African Trypanosomiasis are parasitic diseases which pose a threat to U.S. Military personnel who may be required to operate in tropical and subtropical areas of the world. All drugs which are currently available for use against these diseases are, without exception, less than fully effective or have serious side effects. Accordingly, programs to develop better drugs against these diseases have been initiated, and efforts are being directed toward developing laboratory methods to screen and evaluate chemotherapeutic and chemoprophylactic agents. In-house research is supported and coordinated with contract research.

### A. Development of Antileishmanial Drug Screening Systems

### 1. In vitro System - Axenic Amastigotes:

The  $\underline{\text{in}}$   $\underline{\text{vitro}}$  system for culturing Leishmanial amastigotes axenically which was developed at USAMRU, Panama, is being adapted for use in the antileishmanial drug screening program at WRAIR. A new laboratory has been established and equipped, and personnel trained.

Previously, only certain lots of fetal calf serum (FCS) could be used in the culture system, and prior to the purchase of one large lot, sample aliquots of a large number of lots had to be tested to find one which would support leishmanial growth. In a collaborative effort with Drs. Shiigi and Mishell, Department of Bacteriology and Immunology, University of California, Berkeley, a cryophilic bacterium has been isolated from "good" lots of FCS. The supernatant fraction collected from cultures of these bacteria, when added to a deficient lot of FCS, renders it capable of supporting leishmanial growth in the culture system. Studies are in progress to determine the nature of the growth factor. Currently, all lots of FCS used in the leishmanial culture system are first tested immunologically by the Mishell-Dutton method for presence of the growth factor.

The synthetic stearic acid derivative, glycyl-L-histidyl-L-lysine has been used in other laboratories to support the growth of mammalian cells in culture  $\underline{\text{in}}$   $\underline{\text{lieu}}$  of serum. Addition of as little as 40 nanograms per ml of this material to culture medium does support the growth of large numbers of leishmanial organisms in our culture system without addition of FCS. Further investigations of this FCS substitute are planned.

The culture system has been miniaturized so that it can be used for the screening of large numbers of chemicals for anti-leishmanial activity. The entire cultivation, conversion to amastigote forms, and exposure to test drug can now be carried out in small disposable centrifuge tubes

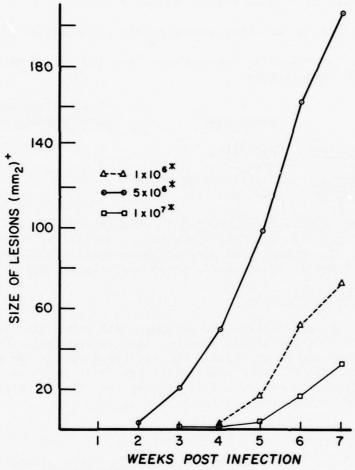
The activity of the standard antimonial antileishmanial drug, glucantime, has been determined in the culture system. An LD  $_{100}$  was reached at a drug concentration of 150  $\mu g$  antimony/ml of media when the amastigotes were exposed to the drug for 96 hours.

### 2. Animal Models of Leishmaniasis

# a. Mystromys albicaudatus (African White-Tailed Rat)

Initial problems of morbidity and mortality which were encountered when this rodent was first introduced into the WRAIR animal colony have been overcome by establishing rigid husbandry standards and by maintaining these animals under strict isolation. Mystromys are highly susceptible to bacterial infections that most other laboratory rodents tolerate well. Studies to characterize Leishmania braziliensis infection in mystromys as a model for New World cutaneous leishmaniasis are continuing. These include definition of the histopathological characteristics of the initial infection, studies of the subsequent development of the cutaneous lesion, and evaluation of the chronic infection.

In mystromys experiments conducted in Panama, glucantime in a regimen of 100 mg/kg per day for 10 days was sufficient to close primary lesions that were 13 weeks old at the time of treatment (see USAMRU Annual Report, 1976). A more rapidly developing primary cutaneous lesion has been produced in mystromys after inoculation of 5 x  $10^6$  amastigotes of L. mexicana (figure 1). In this model, a large papule forms in one week, and an open lesion develops within 4 weeks after inoculation. Treatment with glucantime beginning 4 days after inoculation prevented lesion development in 7 of 10 mystromys given 100 mg/kg for 10 days, and in 2 of 5 mystromys given 100 mg/kg for 4 days. The L. mexicana/mystromys model is advantageous in that the entire test and observation period can be completed in 4 weeks. Evaluation of this model is continuing.



- + Size=Length x With
- \* Each Inoculum is Represented by 5 Animals

Figure 1. Lesion Development of <u>Leishmania mexicana</u> in <u>mystromys albicaudatus</u> inoculated with varying numbers of amastigotes.

It has also been demonstrated that lesions can be produced in mystromys by inoculation of the Jericho vaccine strain of  $\underline{L}$ .  $\underline{\text{tropica}}$ . Ten of 10 animals inoculated with  $10^6$  promastigotes developed cutaneous lesions. These lesions are, however, self-limiting, and healed in 7 of 10 animals within 2 months.

### b. Search for other animal models of cutaneous Leishmaniasis

The following species have been inoculated with promastigotes L. braziliensis:

Species	Common Name		nimals Inocu- oping Lesions
Chinchilla laniger	Chinchilla	5	0
Octodon dagus	Dagu	4	3
Acomys canirinus	Egyptian spiny mouse	4	0
Rattus norvegicus	Nude white rat	4	0

Although lesions were produced in both chinchillas and dagus, the lesions took over one month to develop, and all self-healed within 3 months. It appears that none of these species will serve as useful animal models of new world cutaneous leishmaniasis.

### 3. Cryobank Project

Through acquisition and exchange with other laboratories, a reference collection of lll species, strains and isolates of <a href="Leishmania"><u>Leishmania</u></a> has been accumulated. The reference collection also includes 22 isolates of other hemoflagellates, 5 isolates of <a href="Plasmodia">Plasmodia</a>, 10 <a href="Toxoplasma"><u>Toxoplasma</u></a> and coccidia, and 25 <a href="Leptospira"><u>Leptospira</u></a>. The isolates currently maintained in the cryobank are listed in Table 1.

### 4. Diagnostic Laboratory Services

In July, 1977, a number of suspected cases of cutaneous leishmaniasis were observed in troops of the 82nd Airborne Division returning to Fort Bragg, NC, after field exercises in Panama. A request for epidemiological consultant service was directed to WRAIR. Four culture-positive cases were identified, and recommendations for treatment and future epidemiologic study were made.

# B. Development and Assessment of an In vitro Screen for Potential Antitrypanosomal Drugs

Progress during the past year has included the following:

1. Optimization of pH and duration of culture in PBS: Horse Serum media to 7.35 and 2 hours respectively. During this time the parasites are able to survive and remain actively motile as deter-

mined by light microscopy. Uptake of  $^3\mathrm{H-thymidine}$  and  $^{14}\mathrm{C-glucosa-mine}$  is reliable.

2. Development of a computer program for automatic data analysis. In order to achieve a high potential through-put, it was essential that the raw data in the form of CPM at each dilution of the unknown compounds be input rapidly to a computer for analysis.

The present system is based on a Tektronix 4051 Graphics Computer System. Data (CPM) on teletype punch tape are produced directly from the scintillation counter. This tape is then read sequentially into a preassigned matrix in the computer. Based on standardized quenching curves and an external standardization ratio for each count, the CPM to DPM conversion is calculated for both  $^{3}{\rm H}$  and  $^{14}{\rm C}$ . Each compound has duplicate determinations for a series of 7 two-fold dilutions (64-fold range of concentration).

Initial evaluation of 40 unknown compounds (including some known effective antitrypanosomes) indicated that a simple log transformation of the independent variable—concentration—would result in a linear relationship to DPM in most cases. This information is now incorporated into the program for regression analysis. The regression analysis is performed by standard matrix operations and generates an ANOVA for the significance of the regression and lack of fit to the regression model independently for <sup>14</sup>C and <sup>3</sup>H DPM for each compound. Finally an ED-50 is calculated for the unknown compound based on the projected concentration necessary to cause a 50% reduction in the control DPM calculated from the same plate. The 95% confidence interval for the estimated ED-50 are also calculated by the formula for propagation of variance using the data from each regression analysis.

Any regression yielding a significant lack of fit to the regression model:

### $Y = A + B \log X$

or in which the estimated ED-50 is beyond the range of concentrations tested causes the program to reject that analysis. The system is designed to test routinely concentrations from 12.5 to 0.195  $\mu$ g/ml. The computer program also produces a graphic display of the regression for each test compound.

3. A system was devised for dissolution of compounds in preparation for assay which we have found successful for over a hundred compounds evaluated to date. The compound  $(2.0 \pm 0.1 \text{ mg})$  is first dissolved in 0.5 ml of DMSO. This is then added dropwise to a solution of PBS: Horse Serum (0.26:5.0 ml) with mixing. The compound in PBS: Horse Serum then serves as a stock solution for the unknown compound. The highest concentration of DMSO in the culture wells is less than 0.1%, a level shown to have no effect on the assay system.

- 4. The use of an  $0_2$  microelectrode to assess oxygen utilization in culture wells proved cumbersome and unreliable. This feature of the original design has been abandoned.
- 5. Further refinement of the data analysis program is in progress. This will permit storage of raw data and results for each compound on magnetic tape providing not only efficient storage for a large amount of data, but also permitting later retrieval of data for listing of compounds in relation to their relative activity in the antitrypanosomal assay.
- 6. Though we have been successful in getting uptake of \$\$^{14}\$C-glucosamine by \$\overline{T}\$. rhodesiense in vitro, we have not, to date, been successful in demonstrating that the isotope is incorporated into the glycoprotein coat. Before proceeding further with the use of the assay as a primary screen, further work will be conducted toward establishing whether or not the glucosamine is, in fact, being incorporated into the glycoprotein coat. Currently, it is known that leishmanial and schistosomal species possess enzymes capable of diverting glucosamine into the glycolytic pathways for those organisms. Such information is not now available for trypanosomes.
- If, in the next several months, we are not successful in demonstrating incorporation of glucosamine into the glycoprotein coat of the organism, we intend to return to the use of a  $^{14}$ -C labelled amino acid (probably isoleucine) as a secondary indicator. Though this would not be a specific indicator of surface coat synthesis, it would offer an independent measure of activity by the trypanosome in relation to drug concentration.

# 

A mouse test has been developed to screen chemical compounds for their ability to block skin penetration by <u>Schistosoma mansoni</u> cercariae. A total of 347 specially selected compounds have been tested to date, of which 107 have exhibited antipenetration activity.

Test drugs are dissolved or suspended in alcohol or another vehicle, and the tail of each mouse is suspended for 5 minutes in a test tube containing the drug preparation. After drying, the mice are held for 24 hours at which time the tails are "washed" by immersion in continuously flowing tap water for 30 minutes. One to two hours later the mice are exposed to 100 S. mansoni cercariae by tail immersion for 30 minutes. Vehicle-treated control mice are infected with each drug test group. At 7 weeks after cercarial exposure the mice are killed and the total adult worm burden of each mouse is determined by the portal perfusion technique. A significant reduction (>50%) in worm burden is considered evidence of anti-

penetration activity. Test compounds which have antipenetration activity at 24 hours are retested at progressively longer intervals to determine the duration of persistence.

The 107 compounds which were active in the 24 hour antipenetration-wash test may be grouped into the following chemical classes:

C1a	ss	No. of compounds tested	No. of active compounds
1.	4-Aminoquinolines	60	43
2.	Quinolinemethanols	3	1
3.	Acridines	7	5
4.	Dehydroabietic acid salts	10	6
5.	Hexachlorophene analogs	17	11
6.	Aminothiols a) Polynuclear disulfides (amidine type)	12	6
	<ul><li>b) Aryl disulfides (amidine type)</li></ul>	7	2
	c) Dithiocarbamates	8	4
	d) Thiadiazoles	6	4
7.	Miscellaneous	217	<u>25</u>
	Total	347	107

The 4-aminoquinoline class contained 43 active compounds, the largest number of actives of any of the classes represented. Five of the most active compounds in this group were effective at test concentrations as low as 1.25%. The active quinolinemethanol was the antimalarial drug mefloquine (WR 142490), which was effective at a concentration as low as 2.5%. None of the 4-aminoquinolines protected mice against lethal cercarial challenge (3000 cercariae) when the compounds were administered subcutaneously 24 hours before cercarial exposure; they were active only when applied topically. Mefloquine protected both topically and parenterally.

Hexachlorophene, bithionol and their analogs were the most potent and persistent compounds in the topical test. Hexachlorophene protected against 24 hour challenge at a concentration of only 0.14%; at 1.25% concentration it afforded protection for as

long as 6 days. Bithionol protected for 24 hours at a concentration of 0.42%; at 1.25% concentration it protected for 4 days. Both bithionol and hexachlorophene, when topically applied at 1.25% concentration (with washing), protected against a challenge of 3000 cercariae at 24 hours. The commercial preparation, "Phisohex" (Winthrop Laboratories, NY, NY), which contains 3% hexachlorophene, did not protect in the topical test at concentrations below 2% hexachlorophene. It appears that the alcoholic vehicle used in the screening tests is important in rendering test compounds persistent and resistant to washing.

The dehydroabietic acid salts protected when applied topically at concentrations of 0.74-1.8%. Protection at concentrations of 1.25% did not extend beyond 3 days.

Activity was also noted after topical application of compounds of the acridine, aminothiol, and miscellaneous categories. None of these compounds protected at concentrations below 2.5%, nor were any of them remarkably persistent.

Table 1 - Strain and Stabilate Catalog
Leishmania

# I. Reference Strains

Species	Host	Locality/ Source	Stabilates
L. m. amazonensis	Rodent	Brazil/ Lanson & Shaw	WR-065 WR-067
L. m. amazonensis	Rodent	Brazil/ Lanson & Shaw	WR-021
L. b. guayanensis	Man Sousa	Brazil/ Lanson & Shaw	WR-020
<u>L</u> . <u>b</u> . <u>guayanensis</u>	Man Sousa	Brazil/ Lanson & Shaw	WR-075
L. tropica	Man ATCC	Iran/ ATCC	WR-012 WR-071
L. donovani	Man	Sudan/ ATCC	WR-010 WR-068 WR-124
L. enriettii	Guinea Pig	Brazil/ ATCC	WR-011
L. tropica	Man	Iraq?/ Israel	WR-019
L. tropica (Jerico Vaccine)	Man	Israel	WR-061
L. hoogstraali	Gecko	Sudan/ Israel	WR-056
L. adleri	Lizard	Kenya/ Israel	WR-074
L. donovani	Man	India/ Israel	WR-046
L. donovani	Man	India/ Israel	WR-047

# I. Reference Strains (Contd) Table 1-Strain and Stabilate Caralog

Species	Host	Locality/ Source	Stabilates
L. agamae	Lizard	Israel	WR-097
L. b. braziliensis	Man	Brazil	WR-129
L. donovani (Khartoum)	Man (?)	WRAIR Dr. Stauber	WR-130
L. <u>tropica</u> (major) Seidman	Man (?)	WRAIR Dr. Stauber	WR-131
L. peruviana (Uta)	Man	Peru/Zeladon	WR-140
L. tarantolae	Lizard	Algeria/ Zeladon	WR-144
L. braziliensis	Man/Fisher	Bolivia/ UCLA Med Cen	WR-149 WR-150

## Leishmania

Table 1-Strain and Stabilate Catalog

H = Hamster

M = Man

AT = After Treatment

# II. <u>USAMRU - Panama Isolates</u>

Species	Host/Name Host	Locality/ Area	Stabilates
L. b. panamensis	Man/Arnspiger	C.Z.	WR-057
L. b. panamensis	Man/Otero-Rivera		WR-050
L. b. panamensis	Man/Boynton	c.z./	WR-052
L. b. panamensis	Man/Tomes	Panama	WR-059
L. b. panamensis	Man/L.Perez	C.A./	WR-055
L. <u>sp</u> .	Lu. <u>trapidol</u> Sandfly	Panama/	WR-053
L. b. panamensis	Man/Navarro	Panama/ Pinas Beach	WR-051
<u>L</u> . <u>sp</u> .	<u>Lu. shannoni</u> Sandfly	Panama/	WR-054
L. b. panamensis	Man/Pearlman	C.A./	WR-058
L. b. panamensis	Man/F. Gomez	Panama/	WR-005
L. b. panamensis	Man/Legoas	C.Z./ Empire Range	WR-009
L. b. panamensis	Man/Husbands	C.Z./ Empire Range	WR-003
L. b. panamensis	Man/Marlow	C.Z./ Empire Range	WR-004
L. b. panamensis	Man/Watterson	C.Z./ Chiva Chiva	WR-006
L. b. panamensis	Man/Flores	Panama/	WR-007
L. b. panamensis	Man/Barstow Hamster	C.Z./ Empire Range	WR-060 WR-069

Panama Isolates (Contd) Table 1-Strain and Stabilate Catalog

	Host/Name	Locality/	
Species	Host	Area	Stabilates
L. b. panamensis	Man/Murray Hamster	C.Z./ Ft. Sherman	WR-008 (M) WR-073 (H)
	namster	rt. Sherman	WR-073 (H)
			WR-120 (H)
			WR-128 (H)
			WR-157 (H)
			WR-158 (H)
			WR-064 (H)
L. b. panamensis	Man/Fizer	C.Z./	WR-017 (M)
		Ft. Sherman	WR-070 (H)
			WR-079 (H)
L. b. panamensis	Man/Grutzmacher	C.Z./ Ft. Sherman	WR-001
L. b. panamensis	Man/Carmain	C.Z./ Ft. Sherman	WR-002
L. b. panamensis	Man/Ackerman	C.Z./Lab	WR-062
L. b. panamensis	Man/Deloatch	C.A./ Ft. Sherman	WR-072
L. b. panamensis	Man/Deloatch	C.Z./ Ft. Sherman	WR-077 (AT)
L. b. panamensis	Man/Sablan	C.Z./ Ft. Sherman	WR-076
L. b. panamensis	Man/Sablan	C.Z./ Ft. Sherman	WR-049 (AT)
L. braziliensis (Espundia)	Man/Terborgh	Peru-Amazonia	WR-063 WR-080
L. hertigi	Tropical Porcupine	Panama/ Aguacate	WR-081
L. <u>braziliensi</u> s	Man/Van Sickle	Honduras/ Rio Platanos	WR-084
L. b. panamensis	Man/Murillo	Panama/ Chilibre	WR-085 WR-092

Panama Isolates (Contd) Table 1-Strain and Stabilate Catalog

Species	Host/Name Host	Locality/ Area	Stabilates		
L. b. panamensis	Man/Sablan	C.Z./ Ft. Sherman	WR-099 (ATX2)		
L. braziliensis	Man/Garmine	Costa Rica/ Cahuita	WR-095		
L. b. panamensis	Man/Jonas C.Z./ WR- Ft. Sherman		WR-103		
L. chagasi	Man/Herrera	Honduras/ Cedros	WR-115		
L. chagasi	Man/Salgado	Honduras/ Las Playas	WR-111 WR-113		
L. chagasi	Man/Herrera	Honduras/ Cedros	WR-110		
L. mexicana	Man/(?)	USA/Texas (?)	WR-114		
L. chagasi	Man/Herrera Hamster	Honduras/ USAMRU	WR-116		
L. chagasi	Man/Herrera Hamster	Honduas/ USAMRU	WR-117		
L. chagasi	Man/Salgado Hamster	Honduras/ USAMRU	WR-119		
L. chagasi	Man/Salgado Hamster	Honduras USAMRU	WR-118		
L. chagasi	Man/Herrera Hamster	Honduras/ USAMRU	WR-121		
L. chagasi	Man/Herrera Hamster/Dog	Hondruas/ USAMRU	WR-122		
L. chagasi	Man/Herrera Hamster/Dog	Honduras/ USAMRU	WR-125		
L. mexicana	Man/Hamster	USA/Texas USAMRU	WR-126		

Panama Isolates (Contd) Table 1-Strain and Stabilate Catalog

Species	Host/Name Locality/ Decies Host Area		
	nose	Area	Stabilates
L. mexicana	Man/Hamster	USA/Texas USAMRU	WR-127
L. b. panamensis	Man/Acosta	Panama/Llones	WR-134
L. b. panamensis	Man/Tush	Panama/Cerro Pelado	WR-132
L. b. panamensis	Man/Tush	Panama/Cerro Pelado	WR-133
<u>L</u> . <u>b</u> . <u>panamensis</u>	Man/Hernandez	Panama/ Rio Pequeni	WR-135
<u>L</u> . <u>b</u> . <u>panamensis</u>	Man/Hernandez	Panama/ Rio Pequeni	WR-136 (AT)
L. b. panamensis	Man/Jimenez	Panama/ Empire Range	WR-148 (AT)
L. hertigi	Tropical Porcupine	Panama/ Aguacate	WR-151
L. b. panamensis	Man/ Zimmeriman	Panama/ Empire Range	WR-153
L. b. panamensis	Man/ Hernandez	Panama/ Rio Pequein	WR-154 (AT)
L. b. panamensis	Man/Jecminek	C.Z./ Empire Range	WR-155 WR-156
L. donovani Khartoum	Hamster	Khartoum/WRAIR Med Chem	WR-168
L. donovani HM	Hamster	Sicily/WRAIR Med Chem	WR-163
<u>Leishmania</u> sp.	Man/Lusher	Southern Calif/ USA	WR-164
L. tropica (Jericho)	Mystromys	Israel/WRAIR	WR-178

Panama Isolates (Contd) Table 1-Strain and Stabilate Catalog

Species	Host/Name Host	Locality Area	Stabilates			
Species	nost	Alea	Stabilates			
Leishmania sp.	Man/Llewellyn	Brazil/WRAIR	WR-177			
<u>Leishmania</u> <u>tropica</u> <u>Seidman</u>	Mouse	Senegal/WRAIR	WR-182			
L. m. mexicana	Hamster	B Honduras/ Farrell	WR-183			
<u>L</u> . sp.	Mouse	Rat/Honduras/ WRAIR	WR-194			
<u>L</u> . sp.	Guinea Pig	Rat/Honduras/ WRAIR	WR-195			
<u>L</u> . sp.	Mystromys	?/WRAIR	WR-196			
<u>L</u> . sp.	Man	Van Studen/ South Africa	WR-198			
<u>L</u> . sp.	Hyrax	Johannesburg/ South Africa	WR-199			
<u>L</u> . sp.	Sandfly	Johannesburg/ South Africa	WR-200			
L. mexicana	Mystromys	Texas/WRAIR	WR-201			
<u>L</u> . sp.	Hamster	Rat/Honduras WRAIR	WR-202			

Trypanosoma Table 1-Strain and Stabilate Catalog

		Locality/			
Species	Host	Area	Stabilates		
$\underline{\underline{\mathbf{T}}}$ . sp. and/or	Sloth, 3-Toed	Panama/	WR-086		
Endotrypanum sp.		Aguacate	WR-089		
T. sp. and/or	Sloth, 2-Toed	Panama/	WR-093		
Endotrypanum sp.	broch, 2 roca	Aguacate	WIK-075		
zardoti y paritam op.		Aguacace			
$\underline{\mathbf{T}}$ . sp. and/or	Sloth, 2-Toed	Panama/	WR-094		
Endotrypanum sp.		Aguacate			
$\underline{\mathbf{T}}$ . sp.	Porcupine	Panama/	WR-018		
		Chorrera(?)			
T. cruzi	Man	Brazi1	WR-023		
1. 01011		DIGZII	WR-152		
			WK-132		
T. cruzi	Raccoon	USA/Maryland	WR-022		
1314 Hemoflagellate	Rat	Honduras/	WR-137		
unknown sp.		Tenideros	WR-141		
1310 Hemoflagellate	Opossum	Honduras/	WR-138		
unknown sp.	Opossum	Tenideros	WR-136 WR-145		
diknowii sp.		renideros	WK-143		
1319 Hemoflagellate	Opossum	Honduras/	WR-142		
unknown sp.		Tenideros	WR-146		
1317 Hemoflagellate	Rat	Honduras/	WR-143		
unknown sp.		Tenideros	WR-147		
Endotrypanum ?	Rhesus #3	Panama	WR-087		
Endoctypanum .	Rifesus #3	I allama	WR-090		
			WK-090		
Endotrypanum ?	Rhesus #4	Panama	WR-088		
			WR-091		
MC-009 T. lewisis	Mouse	/WRAIR	WR-189		
			WR-185		

Toxoplasma Table 1-Strain and Stabilate Catalog

Strain	Host/Source	Locality	Stabilates
Miranda	Man/Mouse	Panama	WR-013
CDC - Rh (new)	Mouse	CDC	WR-014
Russell	Man/Lymph node	Panama	WR-015
CDC - Rh (old)	Mouse	CDC	WR-016
Reed	Man/Lymph node	Panama	WR-048
C-37	Mouse	CDC, 1966	WR-82
Quiros	Man/Lymph node	Panama	WR-096
Barrera	Man/Lymph node	Panama	WR-100
M-7741	Mouse	CDC, 1966	WR-102
Isosporo Artopithica	Marmoset	Panama	WR-123

Amoeba Table 1-Strain and Stabilate Catalog

Strain	Species	Source	Stabilates
CCAP 1518-15	Naegleria gruberii	AFIP	WR-108
L1-L	Naegleria gruberii	AFIP	WR-105 (troph) WR-106 (cysts)
CCAP 1518-ID	Naegleria gruberii	AFIP	WR-107
CCAP 1501-1	Acanthamoeba castellanii	AFIP	WR-109
Insect Cell	Line		
<b>Gra</b> ce	Antheraea eucalyptii	WRAIR	WR-066
Plasmodium			
MC-004	P. flaciparum (Smith)	Columbian, WRAIR	WR-167
MC-007	P. inui	Cynomolgus, WRAIR	WR-172
MC-012	P. falciparum (Smith)	Aotus, WRAIR	WR-188 WR-190 WR-191 WR-192

Table 1 - Strain and Stabilate Catalog

Miscellaneous

# Leptospira

Strain	Species	Source	Stabilates
CZ-188	L. canalzonae	WRAIR	WR-024
	L. caniconla hondutrecht	WRAIR	WR-025
HS-622	L. borincana	WRAIR	WR-026
A	L. autumalis akiyama	WRAIR	WR-027
Je-2	L. bratislava	WRAIR	WR-028
CZ-299	L. tropica	WRAIR	WR-029
	L. tarassovi perepelicin	WRAIR	WR-030
CZ-390	L. weaveri	WRAIR	WR-031
LT-821	L. shermani	WRAIR	WR-032
HS-616	L. alexi	WRAIR	WR-033
CZ-320	L. kobbe	WRAIR	WR-034
3705	L. wolffi	WRAIR	WR-035
Patco I	L. biflexa	WRAIR	WR-036
L-16a	L. alexi	Man/Hajduk/Urine	WR-037
L-24a	L. alexi	Man/Hajduk/Blood	WR-038
L-100c	<u>L</u> . sp.	Man/Garzona/Urine	WR-039
L-62a	<u>L</u> . sp.	Man/Garzona/Blood	WR-040
L-46c	<u>L</u> . sp.	Man/Johnson/Urine	WR-041
L-45a	L. sp.	Man/Johnson/ Spinal fluid	WR-042

Table 1 - Strain and Stabilate Catalog
Miscellaneous

# Leptospira

Strain	Species	Source	Stabilates
L-28a	<u>L</u> . sp.	Man/Pratt/Urine	WR-043
L-135a	L. sp.	Man/Neal/Urine	WR-044
L-106a	L. sp.	Man/Lima/Blood	WR-045
L-108a	<u>L</u> . sp.	Man/Becker/Blood	WR-104
L-193	L. sp.	Man/Shobe/Blood	WR-112
L-224	L. sp.	Man/Chandler/Blood	WR-139

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 207 Model Systems for Antiparasitic drugs

## Literature Cited

## Publications:

- Hendricks, L. D. Host range characteristics of the primate coccidian, <u>Isospora arctopitheci</u> rodhain 1933 (Protozoa: Eimeriidae). J. Parasit. <u>63</u>: 32-35, 1977.
- 2. Hendricks, L. D., Wood, D. E., and M. E. Hajduk. New Liquid Media for the Rapid and Quantitative Cultivation of Hemoflagellates. Parasitology. (In press)

Project 3M161102BS01
RESEARCH ON MILITARY DISEASES

					OA O		// 10• (			K&E(AK)030
76 10 01	D. Change	S. SUMMARY SCTY	4. WORK SECURITY	7. REGR.	ADING®	NL	'N INSTR'N	OL SPECIFIC CONTRACTOR	ACCESS	9. LEVEL OF SUM  A. WORK UNIT
10. NO./CODES:	PROGRAM ELEMENT	PROJECT	NUMBER	TASK A	AREA NUM	IBER		WORK UNIT	NUMBER	
& PRIMARY	61102A	3M161102BS	01	00 121						
b. CONTRIBUTING										
c. XXXXXXXXXX	CARDS 114F			<u> </u>						
(U) Ecology and Control of Disease Vectors and Reservoirs  2. SCIENTIFIC AND TECHNOLOGICAL AREAS®										
	ogy 005900 En	vironmenta	1 Biology	01010	O Mic	robio	logy			
The second second second			PLETION DATE		ING AGEN	C¥ .		G Im		
54 09		CONT		DA	L_			C. In-	_	
A DATES/EFFECTIVE:	MA	EXPIRATION:		18. RES	PRECEDIA		& PROFESSI	ONAL MAN YRS	L FUN	DS (In thousands)
b. NUMBER:*	NA	Carina (Ioni		FISCAL	77			5	1 2	234
C TYPE:		4 AMOUNT:			CURRENT				+-	
e. KIND OF AWARD:		f. CUM. AMT.			78		6	5	1 2	276
19. RESPONSIBLE DOD	PREMITATION			20. PERI	FORMINGO					
	gton, DC 2001		esearch	ADDRESS	Div o ∺Wash	f CD& ingto	I n, DC 2	20012		Research
NAME:	Rapmund, CO	L G.		PRINCIPAL INVESTIGATOR (Pumloh SEAN II U.S. Academic Institution)  HAME:  GOULD, Dr. D. J.  TELEPHONE: 202-576-3719						
TELEPHONE:	-576-3551			SOCIAL	SECURITY	ACCOUN	T NUMBER:			
	elligence not			ASSOCIATE INVESTIGATORS Bailey, MAJ C. L.  NAME: Kuenzel, Dr. N.  NAME:						
Vectors; (U	EACH with socially classific (U) Control; (U)	<ol> <li>Taxonomy</li> </ol>								
23. (U) Studies emphasize control of vectors of arbovirus and parasitic diseases of military significance. Objectives are incrimination of vectors and understanding of nost-parasite relationships initially, understanding of vector biology and disease transmission mechanisms ultimately in order to develop more effective control procedures.  24. (U) Invertebrate vectors and vertebrate reservoirs and hosts are collected in areas of known disease activity. Infection rates are determined, as are flight ranges, biological processes, such as pathogen transmission, flight physiology and diapause are										

166

\*Available to contractors upon originator's approval.

DD FORM 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A. 1 NOV 68
AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

DA OA 6431

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

77 10- 01

DD-DR&E(AR)636

Project 3M161102BS01 BASIC RESEARCH ON MILITARY DISEASES

Work Unit 121 Ecology and control of disease vectors and reservoirs

Investigators

Principal: Douglas J. Gould, Ph.D.

Associate: COL Bruce F. Eldridge, MSC; MAJ Charles L. Bailey, MSC;

Douglas M. Watts, Ph.D.; CPT Lyman W. Roberts, MSC; CPT John W. Taylor, MSC; Joel M. Dalrymple, Ph.D.;

Nancy T. Kuenzel, Ph.D.; Tatsuo Hase, Ph.D.

COL Dan C. Cavanaugh, MSC; LTC Paul K. Hildebrandt, VC; David E. Hayes; SP5 Ralph Tammariello; SP5 Michael Richards

### Description

This task involves field and laboratory studies of the relationships between selected arthropods and various aspects of their natural environment, especially those aspects relating to certain pathogenic organisms, their hosts, and their reservoirs. Included are ecological and physiological studies on arthropods, studies of transmission mechanism and the development of improved methods of control of arthropods of medical importance.

# Progress

1. Overwintering Ecology of St. Louis Encephalitis Virus

St. Louis encephalitis (SLE) virus is maintained in nature during the summer and fall by a mosquito-avian-mosquito cycle. At least 3 mosquito species, <u>Culex pipiens</u>, <u>C. nigripalpis</u> and <u>C. tarsalis</u> have been incriminated as vectors of SLE virus. Although the details of summer and fall SLE virus cycle are relatively well known, the mechanism by which the virus persists during the interenzootic winter season is obscure. In temperate regions, <u>C. pipiens</u> and <u>C. tarsalis</u> overwinter solely as inseminated adult females, and some investigators have suggested that SLE virus may persist in hibernating adults of these mosquito species until the next enzootic season. Until the present, there was little convincing evidence to support this hypothesis, however since the 1975 epidemic, we have been reexamining the possibility that SLE virus is maintained in hibernating C. pipiens females during the winter season.

Between January and March 1977, a total of 1162 hibernating C. pipiens females were collected from abandoned ammunition bunkers at several former U.S. Army forts in 4 mid-Atlantic states (Figure 1). The temperatures inside the bunkers were not recorded, but the winter of 1976-77 was one of the coldest on record (Table 1). For the first 7 days following their collection these mosquitoes were held in an insectary at a temperature of 26°C and under a daily photoperiod schedule of 16 hours of light and 8 hours of darkness; at the end of this preliminary holding period they were offered a bloodmeal on a chicken. Those mosquitoes that engorged on chickens were separated from the unfed, and both groups were held in the insectary for varying periods of time until sacrificed for

virus assay. Pools of these mosquitoes (10 mosquitoes each) were inoculated by intracerebral route into 3-5 day old mice, following the virological procedures used during 1976 (WRAIR Annual Progress Report, 1975-76). Two viral isolates were obtained from mosquitoes collected during the period-one from a pool of <u>C. pipiens</u> collected on 26 January at Ft. Washington, Maryland and the second from mosquitoes collected at Ft. Mifflin, Pennsylvania on 22 February (table 2). Presumptive identification based on CF results indicated that both overwintering isolates were similar if not identical to prototype SLE strains (Table 3).

Pre- and post-bleedings of the chickens used to furnish bloodmeals for these mosquitoes were not performed in the processing of the mosquitoes from which the Ft. Washington isolate was obtained. In the case of the Ft. Mifflin isolate, however, such bleedings eliminated any possibility that these chickens could have served as a virus source. Also, virus was not transmitted to the chick by the feeding of the mosquitoes from which the Ft. Mifflin isolate was obtained.

# 2. Overwintering Ecology of Culex pipiens

Overwintering mosquitoes could become infected with SLE virus, either 1) through transovarial transmission or 2) through a viremic bloodmeal taken late in the previous fall. Although transovarial transmission of arboviruses has been demonstrated in mosquitoes, the phenomona has thus far been shown to occur only in mosquitoes which overwinter in the egg stage (<u>i.e.</u> Aedes spp.) and to involve only arboviruses belonging to the California encephalitis group.

The experiments described below were designed to determine whether overwintering Culex pipiens females could have ingested a viremic bloodmeal while in a state of ovarian diapause or "gonotrophic dissociation". Gonotrophic dissociation is defined here to mean failure of ovarian follicles to mature subsequent to a full bloodmeal. In spite of the demonstration of the occurrence of gonotrophic dissociation in Culex mosquitoes in the laboratory (Eldridge, 1966), its occurrence in nature has been questioned because the same environmental conditions, short daily photophases and cool temperatures, which cause ovarian diapause also result in sharply reduced blood feeding. In terms of naturally occurring conditions, however, it seemed a possibility that a population of females which had entered or were about to enter hibernacula might respond to short periods of warm temperatures ("Indian summer") by taking a bloodmeal before entering their hibernacula or by leaving them to take a bloodmeal, but that such warm periods would be of insufficient length to promote ovarian development in all cases. The stock Culex pipiens mosquitoes used in these experiments were reared in our insectary using conventional techniques, and at a temperature of 27°C and a daily photoperiod of L:D 15:9 (hr light: hr dark per 24-hr period). The experimental treatment followed in these studies is diagrammed in Fig. 2. Initially, division of mosquitoes from the stock colony was made at the time of pupation into 2 groups consisting of several hundred pupae each. One

group was placed in an incubator programmed for 15°C and L:D 9:15, the other in an incubator programmed for 15°C and L:D 15:9. Seven days after adult emergence, the short photophase group was further subdivided into 2 subgroups. One of the sbugroups was transferred to another bioclimatic chamber programmed for 25°C and L:D 9:15. The other subgroup was maintained at 15°C and L:D 9:15 as before to constitute a short photophase control. The long photophase group constituted a second control. At this time, and once a day for 7 days, 5 females were removed from each of the 3 groups and dissected in physiological saline. Both ovaries of each female were examined and 5 ovarian follicles measured from each. The length of the follicles was recorded as well as the developmental stage according to the system of Christophers' as modified by Kawai (1969). In addition to the 5 females removed for dissection, 100 females from each group were removed to a 25°C incubator and allowed to feed on a chicken overnight. Fully engorged females from these feedings were held for 10 days, during which time no oviposition substrate was available. Fed females from the long photophase control were held at their pre-feeding conditioning regime: 15:9 L:D, 15°C. Both short-photophase subgroups (i.e., the low temperature controls and the experimental groups which had been transferred to 25°C for varying lengths of time) were held at 9:15 L:D, 15°C. After 10-day holding period, all females were dissected and examined for ovarian development.

The results of the dissections of unfed females are shown in Fig. 3. At the time of the separation of the short photophase females into 2 subgroups, the mean follicle length for these females was 0.031 mm. The long photophase females had ovaries with mean follicle lengths of 0.072 mm. The Christopher stages were No2-N for short photophase females, IIa for long photophase. These stages represent pre-resting and resting stages, respectively. The presence of pre-resting stages at 7 days after adult emergence characterizes ovarian diapause. Follicle lengths for the 2 control groups did not change significantly over a period of 5 days. Females from the short photophase control group had ovaries with follicles which had grown slightly to a mean of 0.036 mm, after 5 days of sampling, or 12 days post-adult emergence. Nearly all follicles at this time were classified as N (pre-resting). Long photophase controls had follicles with a mean length of 0.073 mm at this time.

The experimental group's ovaries gradually grew so that 3 days after being transferred to  $25^{\circ}\text{C}$ , their follicles were the same length and stage as those of the long photophase controls. After 1 day of being held at  $25^{\circ}\text{C}$ , all follicles were at stage I, and after 2 days no follicles had progressed past this stage although there was a mean increase from 0.050 mm to 0.066 mm.

The results of the blood feeding trials and subsequent determination of ovarian development are shown in Table 4. At the time of the first blood feeding trial (day 7 post-emergence) only 5% of the short photophase females took a bloodmeal compared with 78% of the long photophase group. Of those taking full bloodmeals, 80% of the short photophase group displayed gonotrophic dissociation, compared with 0% of the long

photophase group. After 3 days of warming, the percentage of short photophase females taking blood had risen to 45%, but 40% of these showed gonotrophic dissociation. Even after 4 days, when blood feeding took place in 74% of the females, short photophase gonotrophic dissociation occurred in 1/4 of these taking blood. After 5 days, there were no differences between the 2 groups. The suppression of blood feeding and ovarian diapause would appear to be reinforcing phenomena associated with hibernation, rather than 2 antagonistic factors. Furthermore, blood feeding, rather than preventing successful hibernation, would seem to contribute to it.

3. Potential of <u>Aedes sollicitans</u> and <u>Aedes taeniorhynchus</u> for the transmission of Keystone and Jamestown Canyon viruses

Two California encephalitis group arboviruses, Keystone (KEY) and Jamestown Canyon (JC), are enzootic in the Pocomoke Cypress Swamp (PCS) in Southern Maryland. Aedes atlanticus is the principal vector of KEY virus in the PCS in a cycle that involves either gray squirrels and/or cottontail rabbits as vertebrate hosts of the virus. Furthermore, A. atlanticus has been demonstrated to transmit KEY transovarially. During 1976 natural infections of cottontail rabbits with KEY virus on Assateague Island, Virginia were demonstrated, however this is an area where neither A. atlanticus nor gray squirrels are known to occur. Therefore, since Aedes sollicitans and A. taeniorhynchus are two of the most abundant mosquito species on Assateague Island, and assessment of their vector competence was undertaken. Mosquitoes collected from salt marshes near Assateague were fed defibrinated chicken or guinea pig blood-virus mixtures and maintained in the insectary at 25°C for 14 days before being sacrificed for virus assay.

Following ingestion of 3.1  $\log_{10}$ SMICLD<sub>50</sub> of KEY virus, 75% of the A. taeniorhynchus and all of the A. sollicitans became infected (Table 5). The infection rates of A. taeniorhynchus fed on lower concentrations of KEY virus were significantly lower. The small numbers of A. sollicitans available permitted testing at only a single virus concentration.

The quantities of KEY virus recovered from individual mosquitoes after extrinsic incubation periods of up to 14 days are presented in Table 6. The pattern and magnitude of virus recovery differed for the two species even though they ingested comparable concentrations of virus. That multiplication of KEY virus occurred in  $\underline{A}$ . sollicitans is indicated by the average infectivity titers which were greater than 2.7 on day 5 compared to 1.7 on day 0. On days 5 and 7 average infectivity titers for  $\underline{A}$ . taeniorhynchus were lower than for  $\underline{A}$ . sollicitans, but by day 14 virus titers were highest in the former species.

The infection rates of A. taeniorhynchus and A. sollicitans after ingesting JC virus are presented in Table 7. All of the A. sollicitans and A. taeniorhynchus tested became infected after ingesting blood containing 3.5  $18g_{10}$ SMICLD $_{50}$  of JC virus. Infectivity titers observed for

individual A. sollicitans and A. taeniorhynchus after various incubation periods were indicative of virus replication (Table 8).

# 4. Field studies of populations of gray squirrels and Aedes atlanticus

Serological surveillance studies conducted during 1972 and 1973 on the eastern shore of Maryland indicated that infections with Keystone (KEY) virus were occurring in gray squirrels. Subsequent laboratory experiments demonstrated that squirrels develop a viremia following inoculation with KEY virus. In addition, mosquito host preference studies showed that A. atlanticus feeds on squirrels in nature.

A total of 83 different gray squirrels were captured in the Pocomoke Cypress Swamp between May and September 1976. Thirty of these had been trapped previously in 1975. Thirty-one of the 53 (58%) squirrels captured initially in 1976 were negative for KEY antibody when the first blood sample was taken. Eleven of these squirrels (35.5%) were recaptured at least once during the summer. Only one of these squirrels, an adult male, developed serum neutralizing antibody after the initial blood sample. This squirrel seroconverted between 15 July and 29 July 1976, at a time when Aedes atlanticus adults were present in the study area, although in very low numbers. This low seroconversion rate provides an interesting comparison with the 1975 season, when a very large hatch of A. atlanticus occurred. During that year, seroconversion rates in the gray squirrel population followed the A. atlanticus population density very closely. Seroconversion rates increased rapidly in 1975 from 10% during the first week after A. atlanticus emergence to a rate of 70% or greater during the period of peak A. atlanticus abundance. In 1976, no significant hatch of A. atlanticus occurred in the study area, and there was not a significant gray squirrel seroconversion during the season, supporting the hypothesis that A. atlanticus is the primary vector of KEY virus to gray squirrels in the PCS.

### 5. Bionomics of Floodwater Mosquitoes

Field collections of eggs of the floodwater mosquito Aedes canadensis, coupled with laboratory respiration rate studies of diapausing and non-diapausing eggs, were undertaken in an effort to discover the environmental factors inducing embryonic diapause in nature. An understanding of these factors is required in order to forecast mosquito population trends and design control strategies.

Eggs were collected from female Aedes canadensis taken from the Pocomoke Cypress Swamp study area and divided into two treatment groups. One group (non diapausing) was held at 15:9 L:D, 25°C, and a second group (diapausing) at 9:15 L:D, 25°C for 10 days prior to being tested. Embryonic respiration rates for diapausing and non-diapausing A. canadensis embryos were measured using Warburg manometry.

A measurable difference was detected in respiration rates between diapausing and non-diapausing eggs. For example, 1010 eggs which had been held at 15:9 L:D for 10 days and 1025 eggs collected at the same time, but held at 9:15 L:D for 10 days, showed oxygen consumption rates of .0054  $\mu1$   $0_2/\text{eggs}$  per hour and .0042  $\mu1$   $0_2/\text{egg}$ , respectively. When these eggs were flooded with nutrient broth the hatch rate for the 15.9 treatment was 62.9%, compared to 1.7% for the eggs held at 9:15.

# 6. Ecology of Keystone virus in the Pocomoke Cypress Swamp

Transovarial transmission of Keystone (KEY) virus in Aedes atlanticus is an important factor in the perpetuation of that virus in the Pocomoke Cypress Swamp (PCS), especially during the winter season (Le Duc et al., 1975). The infection rate of KEY virus relative to A. atlanticus larval age and factors affecting larval abundance were investigated in 1976 and 1977 in the PCS. During this period parallel studies to assess the effect of temperature on developmental time and survivorship in A. atlanticus were also carried out in the laboratory under controlled conditions. A simulation model of the larval population dynamics of A. atlanticus has been constructed for prediction of larval abundance and for later incorporation into a model of KEY virus prevalence.

Field studies of A. atlanticus were carried out in a 4000 m<sup>2</sup> area of upland forest ("Atlanticus North") adjacent to the western edge of the PCS (Fig. 4). The number of larvae per m<sup>2</sup> of ground pool was estimated using an area sampler modified from the device of Roberts and Scanlon (1974), and the Wilding sampler (Southwood 1966). Air, ground and water temperatures, relative humidity and rainfall were monitored at two points ("Pasture Point" and "Atlanticus East" in Fig. 4) during the period of these studies (Figs. 5, 6). During 1976 rain fell four times on the PCS in sufficient volume to flood A. atlanticus breeding sites and produce egg hatches (Fig. 7), however the water from three of these rainfalls (August 8, 15 and 28) receded before the larvae reached maturity. The larvae that emerged following the rainfall of 3 October failed to complete their development because of cold weather.

Following the October hatch, larval counts were made at 50 to 75 randomly selected locations daily with the aid of the area sampler. Observations were begun when larvae reached the second instar. The number of larvae per  $\rm m^2$  was estimated initially at 120, but the numbers observed declined to  $60/\rm m^2$  and then increased to  $120/\rm m^2$  7 days after observations were begun (Fig. 8). Extrapolating from the area samplings, the population of the whole "Atlanticus North" site was comprised of approximately 320,000 second stage larvae initially, declined sharply during the next two days and then stabilized on 8 October at 120,000 larvae (Fig. 9).

Laboratory studies on the effects of temperature on developmental time were carried out with larvae of  $\underline{A}$ . atlanticus from eggs laid by females collected in the vicinity of Snow Hill, Maryland during August and September 1976. Larvae were were reared under constant temperatures of 19°, 22°, 25° and 26°C and a 15:9 L:D photoperiod. Developmental

time from flooding to adult emergence ranged from 8 days at 26°C to 15 days at 19°C (Table 9). The estimated developmental threshold for these larvae was 14°C, and 14-degree-days above the threshold were required for the completion of each instar. Pupal development velocity was not related linearly to temperature; three days were required to complete pupation at 19°C, but only 2 days at 22°, 25° and 26°C. The larvae reared at 22°, 25° and 26° experienced a constant mortality rate throughout development (Fig. 10), while those maintained at 190C experienced a change in mortality rate at  $41^{\circ}D$  (day 8). The slopes for 9.2 to  $37^{\circ}D$ (days 1-7) and 41 to  $80^{\circ}$ D (days 8-15) are -0.0423 ( $r^2=0.99$ ) and -0.0102 $(r^2=0.97)$ , respectively, and are significantly different from each other (P < 0.01).  $41^{\circ}D$  is the first time during the experiment when numbers of fourth instars exceeded numbers of third instars. Therefore, at 19°C and described experimental conditions, the mortality rate per degree day of instars 1 to 3 is approximately four times that of fourth instars and pupae.

Survivorships for each stage and total immatures at the four constant temperatures are summarized in Table 10. Survivorships ranged from 0.57 for first instars at 22°C to 1.00 for fourth instars at 25°C. Survivorships for instars 1 and 2 at 19°C are approximately 0.25 lower than for older age classes. The first two age classes also exhibit lowest survivorship of mean survivorship for each age class. Calculated survivorship for first instar to adult compares well with observed values. Survivorship of second to third instars in the field was 0.16, a value much lower than the average of 0.74 observed in the laboratory. Field survivorship would be anticipated to be lower, however, since larvae were exposed to predation, crowding and other environmental insults not encountered in the laboratory. Crowding appears not to have been detrimental to larval abundance at observed field densities, however, unless it contributed to the high mortality rate of second instars. After a sharp drop in numbers of larvae per m<sup>2</sup> (Fig. 9) the numbers of larvae in the study area stabilized by 7 October (Fig. 9) while number of larvae per m<sup>2</sup> increased. As area flooded declined (Fig. 7) the density of larvae per m<sup>2</sup> increased by 12 October to the initial observed value.

Minimum infection rates (MIR) virus for different aged  $\underline{A}$ . atlanticus are summarized in Table 11. These rates are based on microtiter cell culture and plaque assay results only. One isolation was made from adult females but none from males. The MIR in fourth instars is significantly larger than that of second instars (p<0.05). However, this difference may result from the variation in pooling methods for second and fourth instars. The minimum infection rate for combined larvael, 0.00131, is similar to that of 0.00193 previously reported for third and fourth instar  $\underline{A}$ . atlanticus collected in the PCS in 1973 (LeDuc et al 1975). In 1975, adult female  $\underline{A}$  edges atlanticus were collected by light trap every two days in the PCS and pooled for virus assay (1975-76 Annual Report, Dept. of Entomology). Eighty virus isolations were made. To date 20 of these isolates have been tested and identified by PRNT as KEY virus (Table 12).

A simulation model of <u>Aedes atlanticus</u> larval population dynamics has been constructed to facilitate the organization of research, hypothesis testing and prediction of larval abundance. The model has been designed as a module which could later be incorporated into a comprehensive model of KEY virus prevalence.

For the construction of this model, the larval  $\underline{A}$ . atlanticus population was viewed as represented in (Fig. 11). The objective of the model is to simulate the number of individuals in each age class per km<sup>2</sup> of breeding area. The parameters used for input into the model were:

- 1) number of age classes
- 2) degree-day increment
- 3) survivorship through an age class
- 4) mortality rate for each age class
- 5) developmental time of each age class in degree-days above threshold
- 6) developmental temperature threshold for each age class
- 7) maximum number of degree-days and real days of desired simulation
- 8) mean environmental temperature for each day of simulation
- 9) rainfall for each day of simulation
- 10) initial area flooded in m<sup>2</sup>
- 11) m<sup>2</sup>/day drying

Basic assumptions were that 1) thermal constants determined in the laboratory apply in the field and, 2) considering survivorship within an age class constant is a reasonable approximation to reality.

The model has been programmed in FORTRAN IV for the CDC 3500 computer. Every 5 degree-days the program outputs 1) cumulative degree-days above threshold 2) cumulative days in real time and 3) number of square meters of breeding area flooded and 4) number of mosquitoes in each age class/ $m^2$  of flooded breeding area and per km of total breeding area.

The general equations used are of the form

$$x_{j}(t+1) = \{I - \phi + x_{j}(t)\}C$$

where  $x = number of individuals/m^2$ 

- j = age class
- t = time in degree days above threshold
- I = number entering age class j
- $\phi$  = number leaving age class j
- C = the factor by which larvae are concentrated or diluted due to changing pool size.

The number entering a stage,  $x_j$ , results from maturation of individuals in the previous stage,  $x_i$   $I_j = s_i x_i (t-K_i)$  where  $s_i$  is survivorship rate of  $x_i$  to  $x_j$ ; t is time in cumulative degree-days  $> t_0$  and  $K_i$  is the thermal constant for stage i. The notation  $(t-K_i)$  means that only those individuals that have been in age class i for  $K_i$  degree-days are ready to mature to age class j. Developmental time lags are generated by this mechanism.

 $\phi_j$  = the number of individuals in age class j old enough to mature to the next age class plus those dying in age class j.

$$\phi_{j} = x_{j} (t-K_{j}) (s_{j} + u_{j})$$

where  $s_j$  is survivorship of stage j and  $u_j$  is mortality rate. This equation states that only those individuals which have been in stage j for  $K_j$  degree-days are ready to mature to the succeeding age class. Also, mortality in age class j falls entirely on the oldest group of individuals.

c = (pool area at t)/(pool area at t + 1)

The equation, then, for the number of indivuals per  $m^2$  for any instar, j, at time t+1 is

$$x_{j}(t + 1) = \{s_{i} x_{i} (t-K_{i}) - x_{j} (t-K_{j}) (s_{j} + u_{j}) + x_{j}(t)\}c$$

Currently the model is being validated for laboratory constant temperature studies and will be further tested with data from the Pocomoke Cypress Swamp.

### 7. Studies of trombiculid mite vectors of rickettsioses

Since October 1975, laboratory studies have been conducted to determine the mite-host-rickettsial interactions required for transmission of Rickettsia tsutsugamushi from one mite generation to the next and from mite to vertebrate host. Additionally, variation in the rickettsial strains transmitted by laboratory colonies of Leptotrombidium spp. are being evaluated (Table 13), and infected mites have been used to provide a "natural challenge of candidate vaccines in laboratory animals. The stylostomes (feeding tubes) of these mites were studied to determine the histological changes in parasitized mouse skin and investigate the possible importance of stylostome characteristics to transmission of R. tsutsugamushi.

Unengorged larvae were applied to the ears of white mice which were sacrificed at periodic intervals. Infested ears were excised, fixed, and serially sectioned. The resulting sections were stained either with hematoxylin and eosin or Giemsa stain.

At 24 hours after attachment of trombiculid larvae to the ears of white mice stylostomes were well formed. Based on distinctive morphologic differences, it was possible to classify these stylostomes into 3 types (Fig. 12). An epidermal stylostome (Fig. 13) was formed by L. intermedium larvae. It was a cone-shaped structure consisting of a central canal, an inner wall of chromophobic materials and an outer wall of incorporated host epidermal tissue. This type of stylostome did not extend beyond the epidermal-dermal junction. The larvae of L. fletcheri formed a mesenchymal stylostome (Fig. 14). This type of stylostome seemed to evoke a marked chemotactic effect on leukocytes and was completely surrounded by a dense aggregate of inflamatory cells. An extension of this feeding tube penetrated the dermal layer along with the sheath of inflamatory cells. The mixed type of stylostome was characteristic of L. arenicola and

L. deliense larvae. This stylostome was similar to the epidermal type in the epidermal portion and showed characteristics of the mesenchymal stylostome in the dermal portion (Fig. 15). Dermal inflamation seen in the mixed stylostome appeared to be intermediate in severity between those of the epidermal and mesenchymal stylostomes.

Lesions resulting from the feeding of R. tsutsugamushi-infected and noninfected L. fletcheri were compared. Both produced typical mesenchymal stylostomes, and the presence of R. tsutsugamushi in the chigger did not appear to alter the histologic picture at the site of feeding. Small particles resembling rickettsiae were observed in large mononuclear cells adjacent to stylostomes of infected larvae in Giemsa-stained sections.

The only species studied that produced an epidermal stylostome, viz. L. intermedium, has never been shown to transmit R. tsutsugamushi. L. fletcheri, L. arenicola and L. deliense, all proven vectors, form mesenchymal or mixed types of stylostomes. The induction of inflammatory changes in tissues adjacent to the feeding tube seemed favorable both for uptake of rickettsiae from infected hosts and for rickettsial transmission to the host. Since the mesenchymal and mixed stylostomes caused more severe inflammation, penetrated deeper into the host tissue and left residual granulomatous foci, species producing these types of stylostomes would appear, on an anatomical basis, to have a greater vector capacity.

There is increasing evidence that the mite is the host, vector and reservoir of scrub typhus. Infected mites represent a very different reservoir of scrub typhus. Infected mites represent a very different rickettsial maintenance system from that of culture media or vertebrate hosts. Marked physiological changes take place during the six life stages of the mite. Also, each mite stage assumes the approximate temperature of its microenvironment. It is not unreasonable, therefore, to expect some variations among artifically passed and naturally maintained strains of  $\underline{R}$ .  $\underline{t}$  sutsugamushi.

During this reporting period, infected L. arenicola invariably transmitted lethal R. <u>tsutsugamushi</u> infections to C3H mice (Table 14). While the exact quantity of rickettsiae transmitted by an individual chigger has not been determined, a dose response based on the number of infected larvae per mouse was observed.

By contrast, the strain of  $\underline{R}$ . tsutsugamushi in the infected  $\underline{L}$ . fletcheri colony failed to kill C3H mice infected by larval feeding. Up to 50 infected larvae were applied to an individual mouse to achieve an increased dosage of rickettsiae but no lethal infection was established.

The strains of R. tsutsugamushi in both infected mite colonies were found to be a very similar to the classical Karp strain by fluorescent antibody typing (Robinson et al, In press; Robinson, personal communication). A TA763 strain component was also present in the strain carried by L. arenicola (Robinson et al, In press).

Preliminary comparisons were made of the 2 mite strains of rickett-siae transmitted by larval feeding using cross protection tests (Table 15). Five infected L. arenicola larvae were allowed to engorge on each of 12 C3H mice. From day 8, the mice were given prophylactic chloromycetin at a rate of 2.5 mg/ml in their drinking water for 21 days. Seven days after the treatment was discontinued, the mice were challenged with a standard dose ( $10^{3-4} IPLD_{50}$  of the Karp strain grown in yolk sack suspension. All of the mice survived. Five L. fletcheri larvae from the infected colony were fed on each of 10 C3H mice. After 21 days, these mice were challenged by feeding 5 L. arenicola larvae from the infected colony on each mouse. Two mice died of nonspecific causes relating to necessary laboratory handling before the L. arenicola larvae had fed to repletion. The remaining 8 mice survived and were challenged a second time with standard Karp 42 days after the initial L. fletcheri feeding. All 8 of the mice survived.

In a preliminary evaluation of an experimental vaccine which included the standard Karp strain, 5 infected  $\underline{L}$ .  $\underline{\text{arenicola}}$  larvae were fed on each of 10 vaccinated mice. None of these mice survived, although 10 other control mice vaccinated with the same vaccine survived a simultaneous standard Karp challenge.

These preliminary experiments confirm the original field observations of earlier workers (Irons, 1946; Rights and Smadel 1948) that strains of R. tsutsugamushi vary greatly from one locale to the next. This variation provides a plausible explanation for the failure of early experimental vaccines to protect test subjects exposed to natural infection in the field by strains not contained in the test vaccines.

Project 3M161102BS01 BASIC RESEARCH ON MILITARY DISEASES

Work Unit 121 Ecology and control of disease vectors and reservoirs

# Literature Cited

# Reference:

- 1. Eldridge, B.F. Environmental control of ovarian development in mosquitoes of The <u>Culex pipiens</u> complex. Science 151: 826-828, 1966.
- 2. Kawai, S. Studies on the follicular development and feeding activity of females of <u>Culex</u> tritaeniorhynchus with special reference to those of autumn. Trop. Med. 11: 145-169, 1969.
- 3. LeDuc, J. W., W. Suyemoto, B. F. Eldridge, P. K. Russell and A. R. Barr. Ecology of California encephalitis viruses on the DEL MAR VA Peninsula. II. Demonstration of transovarial transmission. Am. J. Trop. Med. Hyg. 24: 124-126, 1975.
- 4. Robinson, D. M., L. W. Roberts, A. L. Dohany, E. Gan, T. C. Chan, and D. L. Huxsoll. Virulence and antigenic properties of <u>Rickettsia</u> tsutsugamushi in a naturally infected laboratory colony of <u>Leptrotrombidium</u> (<u>Leptotrombidium arenicola</u>. S.E. Asian J. Trop. Med. Pub. Hlth. (in press).
- 5. Irons, E. N. Clinical and laboratory variation of virulence in scrub typhus. Am. J. Trop. Med. 26: 165-74, 1946.
- 6. Rights, F. L. & J. E. Smadel. Studies on scrub typhus (tsutsugamushi disease). III. Heterogenicity of strains of R. tsutsugamushi as demonstrated by cross vaccination studies. J. Exper. Mbd. 87: 339-51, 1948.
- 7. Roberts, D. R. and J. E. Scanlon. An area sampler for collecting mosquito larvae in temporary woodland and field pools. Mosq. News. 34: 467-468, 1974.
- 8. Southwood, T. R. E. Ecological Methods. Methuen & Co. Ltd. London. 391 pp., 1966.

# Publications:

- 1. Eldridge, B. F. Mosquito Ecology, Field Sampling Methods, by M. W. Service. Book Review. Bull. Ent. Soc. Amer. 22: 435-436, 1977.
- 2. Eldridge, B. F., M. D. Johnson and C. L. Bailey. Comparative studies of two North American mosquito species, <u>Culex resturans</u> and <u>Culex salinarius</u>: Response to temperature and photoperiod in the laboratory. Mosq. News 36: 506-513, 1976.
- 3. Hayes, D. E., and R. A. Ward. Sporozoite transmission of falciparum malaria (Burma-Thau. Strain) from man to Aotus monkey. Amer. J. Trop. Med. & Hyg. 26: 184-185, 1977.
- 4. Kuenzel, N. T. Population dynamics of protelean parasites (Hymenoptera: Aphelinidae) attacking a natural population of <u>Trialeurodes packardi</u> (Homoptera: Aleyrodidae) and new host records for two species. Proc. Ent. Soc. Wash. 79: 400-404, 1977.
- 5. Kuenzel, W. J. and N. T. Kuenzel. Basal metabolic rate in growing chicks <u>Gallus domesticus</u>. Poultry Sci. <u>56</u>: 619-627, 1977.
- 6. Pinger, R. R., and B. F. Eldridge. The effect of photoperiod on diapause induction in <u>Aedes canadensis</u> and <u>Psorophora ferox</u> (Diptera: Culicidae). Ann. Ent. Soc. Amer. 70: 437-441, 1977.
- 7. Roberts, L. W. and D. M. Robinson. Efficiency of transovarial transmission of <u>Rickettsia tsutsugamushi</u> in <u>Leptotrombidium arenicola</u>. J. Med. Entomol. 13: 493-96, 1977.
- 8. Roberts, L. W., G. Rapmund and F. C. Cadigan Jr. Sex ratios in Rickettsia tsutsugamushi infected and noninfected Leptotrombidium (Acari: Trombiculidae) J. Med. Entomol. 14: 89-92, 1977.
- 9. Ward, R. A. Culicidae. In: <u>Biota Acuatica de Sud America</u>
  Austral. (S. H. Hurlbert, ed.). San Diego State Univ. Press. pp. 268-274, 1977.
- 10. Ward, R. A. Recent changes in the epidemiology of malaria relating to human ecology. Proc. 15th Int. Congr. Ent., Washington, D.C. pp. 523-529, 1977.

TABLE 1

Average monthly minimum and departure from normal temperatures (OF) recorded at Washington National Airport (10 miles from Ft. Washington) and Philadelphia International Airport (1 mile from Ft. Mifflin) from December 1976 to February 1977

Wash. National Airport	Average minimum 27.1	December 1976 rage Departure ifmum from normal 27.1 -1.9	January 1977 Average Department from 17.5	y 1977  Departure from normal -10.2	Februa Average minimum 27.9	Rebruary 1977 age Departure mum from normal
Philadelphia Airport	22.2	6.4-	12.3	-12.3	24.1	-0.3

Table 2. Hibernating <u>Culex pipiens</u> collected at abandoned Army Forts located in Pennsylvania, New Jersey and Maryland during the winter of 1977.

Mosquit	Mosquito Collection	uo		Labor	Laboratory Handling	20	
				Bloodfed	dfed	Non B1	Non Bloodfed
Location	Date	Number Collected	Days held in insectary	No. Pools Tested	No. Isola- tions	No. Pools Tested	No. Isola- tions
Ft Washington, MD	4 Jan	16	21	3	0	7	0
Ft McHenry, MD	5 Jan	6	7	0	0	1	0
Ft McHenry, MD	11 Jan	3	0	0	0	7	0
Ft Armistead, MD	12 Jan	33	20	2	0	-	0
Ft Washington, MD	26 Jan	215	20	14	1	7	0
Ft Miffiln, PA	22 Feb	907	15	25	1	15	0
Ft Mott, NJ	23 Feb	226	14	10	0	12	0
Ft Mott, NJ	1 Mar	173	20	æ	0	6	0
Totals		1162		62	2	53	0

TABLE 3

Serological Identification of SLE Virus Strains Using Complement-Fixation (CF) Tests

Virus Strain		Antibody HMAF	MAF	
Overwintering Mosquito Isolates	Ft. Wash #7	Ft. Miff #34 CF	Parton <sup>d</sup> CF	Local 1975 CF
Ft. Washington #7	256 <sup>c</sup>	1024	512	256
Ft. Mifflin #34	128	1024	512	512
Reference SLE Virus Strains				
Parton Strain SLE <sup>a</sup>	256	512	512	256
Local 1975 Mosquito Isolate <sup>b</sup>	256	1024	512	512

 a - Research Reference Reagent V-524-001-522 NIAID, suckling mouse passage 3.
 b - An SLE isolate obtained from a midsummer light trap collection of Culex pipiens during the 1975 outbreak. Collections were from the Wash. D.C. area and antigen was prepared from low mouse brain passage material.

- Reciprocal of HMAF dilution exhibiting <50% lysis at an optimal antigen dilution using 5 units of complement,

d - Research Reference Reagent.

TABLE 4

Bloodfeeding and ovarian development in female <u>Culex pipiens</u> exposed to diapause-inducing conditions (L:D 9:15, 15°C) from pupal stage to 7th day of adult life, then warmed to 25°C for from 1 to 7 days

	Experimen L:D 9:15,	Experimental Group D 9:15, 15°C 25°C	Short Photol	Short Photophase Control L:D 9:15, 15°C	Long Photophase Co L:D 15:9, 15 <sup>o</sup> C	Long Thotophase Control L:D 15:9, 15°C
Day of Adult Life	Percent took full blood meal	Percent showing gonotrophic dissociation	Percent took full blood meal	Percent <sup>2</sup> showing gonotrophic dissociation	Percent took full blood meal	Percent showing gonotrophic dissociation
1	S	80.0	1	71.4	7.8	0.0
•	18	61.1	9	83.3	70	0.0
6	21	52.4	S	0.09	79	0.0
10	45	40.0	<b>60</b>	75.0	73	0.0
n	72	25.7	9	50.0	82	0.0
12	19	1.6	6	55.5	11	0.0
13	42	0.0	9	9.99	9/	0.0
41	п	0.0	5	80.0	•	•

| Each group comprised of 100 females

20f those taking full blood meal

TABLE 5

Infection rates of mosquitoes after ingesting Keystone Virus

Mosquito Species	Virus Dose <u>l</u> /	Extrinsic Incubation Period (Days)2/		ction ate
A. taeniorhynchus	3.1	5, 7, 14	9/12	(75%)
	2.0	7, 14	1/8	(13%)
	1.8	5, 7, 14	1/10	(10%)
A. sollicitans	3.1	5, 7, 14	12/12	(100%)

<sup>&</sup>lt;sup>1</sup>Log<sub>10</sub>SMICLD<sub>50</sub>/.03 m1

<sup>&</sup>lt;sup>2</sup>Extrinsic incubation period, approximately same number of mosquitoes assayed on each day

TABLE 6

Keystone virus infectivity titers determined for individual mosquitoes

Mosquito	Virus	Extri	sic Incu	bation Per	iod (Days)	)
Species	Dose1/	0	3	5	7	14
A. taeniorhynchus	3.1	1.4(4)2/	1.4(3)	0.6(1)	1.2(4)	3.0(2)
A. sollicitans	3.5	1.7(4)	2.5(4)	2.7(4)	2.5(4)	1.9(4)

 $<sup>\</sup>frac{1}{L_{\text{log}_{10}}}$ SMICLD<sub>50</sub>/03 m1

<sup>2/</sup>Average infectivity titer,  $\log_{10} \text{SMICLD}_{50}/03 \text{ ml}$ , ( ) number of individual mosquitoes tested.  $\log_{10} \text{dilutions}$  of mosquito suspension were made through  $10^{-3}$  at which point 100% of the mice died for some specimens, thus the SMICLD  $_{50}$  was greater than the values presented.

TABLE 7

Infection rates of mosquitoes after ingesting Jamestown Canyon virus

Mosquito Species	Virus Dose1/	Extrinsic Incubation Period (Days)2/	Infection Rate
A. taeniorhynchus	3.5	5, 7, 14	12/12 (100%)
(4)4) Z (4)4(4)	3.0	5, 7, 14 7, 14	3/5 )60%)
A. sollicitans	3.5	5, 7, 14	12/12 (100%)

 $<sup>\</sup>frac{1}{4}$ Log<sub>10</sub>SMICLD<sub>50</sub>/.03 m1

<sup>2/</sup>Extrinsic incubation period, approximately same number of mosquitoes assayed each day.

TABLE 8

Jamestown Canyon virus infectivity titers determined for individual mosquitoes.

Mosquito	Virus	Ex	trinsic Inc	ubation Pe	riod (Days)	
Species	Dose1/	0	3	5	7	14
A. sollicitans	3.5	1.9(4)2/	>2.3(4)	>2.3(4)	3.2(4)	>2.8(4)
A. taeniorhynchus	3.5	2.4(4)	1.3(4)	1.5(4)	2.8(4)	>3.0(4)

 $<sup>^{1}\</sup>mathrm{Log}_{10}\mathrm{SMICLD}_{50}/\mathrm{03~m1}$ 

 $<sup>^2\</sup>text{Average infectivity titers, }\log_{10}\text{SMICLD}_{50}\text{$\angle 03 ml, ( ) number of individual mosquitoes tested}$ 

TABLE 9

Developmental Time of <u>Aedes atlanticus</u> at Four Constant Temperatures

				*D	evelopme	ntal Tim	e (Days)	
Mean	Number of	Mosquitoes		In	star			
Temp.	Day 2	Final	1	2	3	4	Pupa	Total**
19.3	268	53	2	3	3	3	3	15
22.4	93	17	3	1	2	2	2	11
25.2	60	15	2	1	2	2	2	10
26.2	71	19	1	1	1	1	2	8

<sup>\*</sup> Measured as time between maximum numbers per day of adjacent stages

<sup>\*\*</sup> Measured from time of flooding to adult emergence

TABLE 10
Survivorship\* of Immature Aedes atlanticus at Four Constant Temperatures

Temp	In	star				Total	Observed
(oc)	1	2	3	4	Pupa	Immatures	Total
19	0.645	0.600	0.894	0.884	0.909	0.194	0.20
22	0.569	0.797	0.869	0.849	0.849	0.179	0.18
25	0.833	0.833	0.833	1.000	0.809	0.319	0.25
26		0.718	0.793	0.759	0.806	0.278	0.27
x	0.682	0.737	0.848	0.873	0.843	0.243	0.22
s <sub>x</sub>	0.202	0.103	0.042	0.100	0.048	0.067	0.042
•							

<sup>\*</sup> Survivorship =  $Si^{Ki}$  where s = survivorship per  $^{O}D>14^{O}C$  for stage i, and K = the thermal constant for stage i

TABLE 11

Minimum Infection Rates of Keystone Virus in Aedes atlanticus larvae and adults in the Pocomoke Cypress Swamp in 1976 1

Age	Collection Dates <sup>2</sup>	Number Tested	No. of Pools <sup>3</sup>	No. Pos. Pools	mir <sup>4</sup>
Instar 2	12-21 Aug	4850	194	5	0.001031**
Instar 3	12 Aug	1850	74	1	0.00054
Instar 4	14-19 Aug 14-15 Oct	1725	69	5	0.002901**
Total Larvae		8425	337	11	0.00131
Adult 99	19-27 Aug 14-15 Oct	925	37	1	0.001081
Adult dd	19-27 Aug 14-15 Oct	1225	49	0	0

 $<sup>^{\</sup>mathbf{1}}\mathbf{B}$ ased on microtiter screening and plaque assay of second SMB passage

 $<sup>^{2}\</sup>mathrm{Date}$  of collection for larvae; date of emergence in lab for adults

 $<sup>^{3}</sup>$ Number of mosquitoes/pool = 25

 $<sup>^{4}</sup>$ MIR = No. pos. pools/No. Mosquitoes tested

<sup>\*\*</sup> Different from each other at the 5% level of significance

TABLE 12

Keystone Virus Isolations from <u>Aedes</u> <u>atlanticus</u> adult females light-trapped in 1975

	lection Oate	No. A. atlanticus Tested	No. Virus Isolations	No. KEY Pos. Pools No. Pools Tested
Ju1y	22-26	18450	23	10/10
	28	9300	11	5/5
	30	6250	6	1/1
Aug	1	1850	2	1/1
	3-5	7050	6	3/3
	7-21	12660	29	1/1
Aug 2	3-Sept 18	3250	1	1/1
Sept	20-Oct 24	4474	3	0

TABLE 13

Colonies of <u>Leptotrombidium</u> Studied During FY 76

Species	Country of Origin	Vector Status	Remarks
. fletcheri*	Malaysia	+	Not infected with R. tsutsugamushi
. fletcheri*	Malaysia	+	Infected with R. tsutsugamushi
. arenicola*	Malaysia	+	Not infected with R. tsutsugamushi
. arenicola*	Malaysia	+	Infected with R. tsutsugamushi
. deliensis*	Malaysia	+	Non-infected
. intermedium**	Japan	?	Non-infected

<sup>\*</sup> Obtained from the United States Army Medical Research Unit in Malaysia (Rapmund et al. 1969; Roberts and Robinson, 1977)

<sup>\*\*</sup> Obtained from Dr. R. Traub, Univ. of Maryland, Baltimore, MD

TABLE 14

Virulence of the Leptotrombidium arenicola (LA) strain of R. tsutsugamushi for C3H/HE mice\* exposed to chiggers in laboratory feedings

1 L. arenicola/mouse***	Day of death	15	16	17	18		16.8
1 L. aren	No. of mice	1	9	4	3		11
5 L. arenicola/mouse**	Day of death	12	13	15	17	18	14.9
5 L. aren	No. of mice	က	1	9	2	2	14
nicola/mouse**	Day of death	12	13	14			13.0
10 L. arenicola/	No. of mice	1	1	1			3

Purchased from Flow Laboratories, P.O. Box 1065, Dublin, VA 24084 Some fully engorged chiggers were recovered from each mouse An engorged larva was recovered from each mouse considered in this group.

TABLE 15

Preliminary comparisons of pristine strains of Rickettsia tsutsugamushi maintained in vivo by Leptotrombidium arenicola and Leptotrombidium fletcheri

Strain	Antigenic phenotype	Method of vaccination of C3H mice	Challenge	Results (No. dead per No. Challenged)
L. arenicola	Karp, TA763*	L. <u>arenicola</u> feeding + prophylaxis	Karp***	0/12
L. fletcheri	Karp*	L. fletcheri feeding (no prophylaxis)	L. arenicola feeding	8/0
Karp (ys susp.)	Karp	Experimental vaccine**	L. arenicola	10/10
Karp (ys susp.)	Karp	Experimental vaccine**	Karp	0/10

Fluorescent antibody typing (Robinson et al 1977). Prepared by MAJ  $\rm G_3$  Elsenberg, Rickettsial Disease Section, WRAIR Challenge dose 10  $^{\circ}$  (IPLD  $_{50})$ \* \*

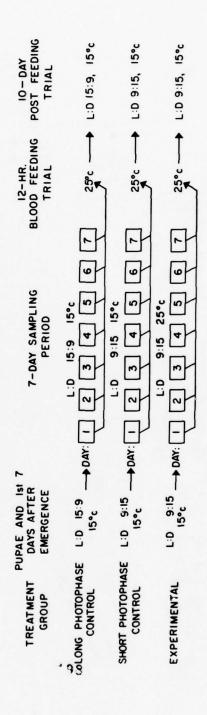
# Captions for Figures

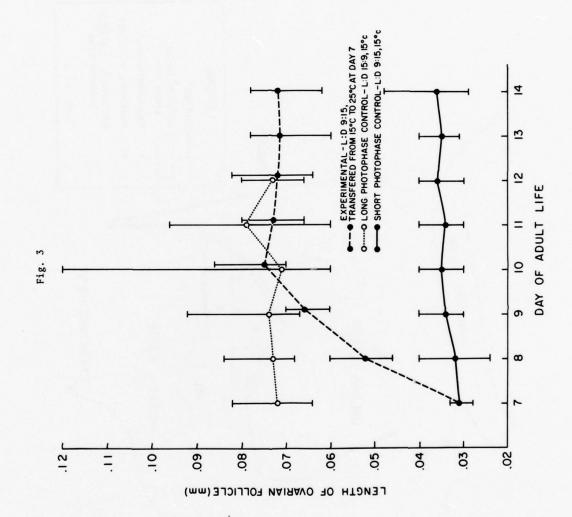
- Figure 1. Location of six former U.S. Army Forts where overwintering mosquitoes were collected.
- Figure 2. Diagram of experimental treatment schedule used to determine effects of photoperiod and temperature upon ovarian diapause in Culex pipiens.
- Figure 3. Length of ovarian follicles of non-blood-fed <u>Culex pipiens</u> females 1 to 7 days after transfer from conditions of L:D 9:15 15°C to L:D 9:15, 25°C. Long and short photophase controls maintained at 15°C continuously. Ranges and means of lengths of 5 follicles from each of 5 mosquitoes shown.
- Figure 4. Map of Pocomoke Cypress Swamp Study Area, Worcester County, Maryland.
- Figure 5. Mean weekly air temperature at Pasture Point in 1976.
- Figure 6. Summary of rainfall, water temperature and Aedes atlanticus hatches in Aug Oct, 1976 in PCS. Larvae histograms indicate presence of specified stage only, not abundance.
- Figure 7. Surface area flooded at "Atlanticus North" after 2 major rainfalls in 1976.
- Figure 8. Number of Aedes atlanticus larvae per m² at "Atlanticus North" in October, 1976.
- Figure 9. Total number of A. atlanticus larvae in the 4000m<sup>2</sup> study area at "Atlanticus North" in October 1976.
- Figure 10.  $\log_{10}$  total numbers of <u>A</u>. <u>atlanticus</u> relative to accumulated degree-days >14°C in four constant temperature experiments.
- Figure 11. Schematic of life system of <u>Aedes atlanticus</u> larvae. Blocks are numbers of larvae in a specified area. Arrows represent movement of individuals. The area within the dotted line is the larval life system.
- Figure 12. Types of stylostomes (feeding tubes) produced by <u>Leptotrombidium</u> spp. larvae: The epidermal stylostome of <u>L</u>. <u>intermedium</u>, the mesenchymal stylostome of <u>L</u>. <u>fletcheri</u> and the mixed stylostome of <u>L</u>. arenicola and <u>L</u>. deliense are illustrated.
- Figure 13. An epidermal stylostome of <u>Leptotrombidium</u> intermedium 24 hr. after attachment on a mouse. a. central canal; b. chromophobic tube c. epidermal coat d. hyperplastic epidermis. e. tissue space at the epidermal-dermal junction. Hematoxylin and eosin stain.

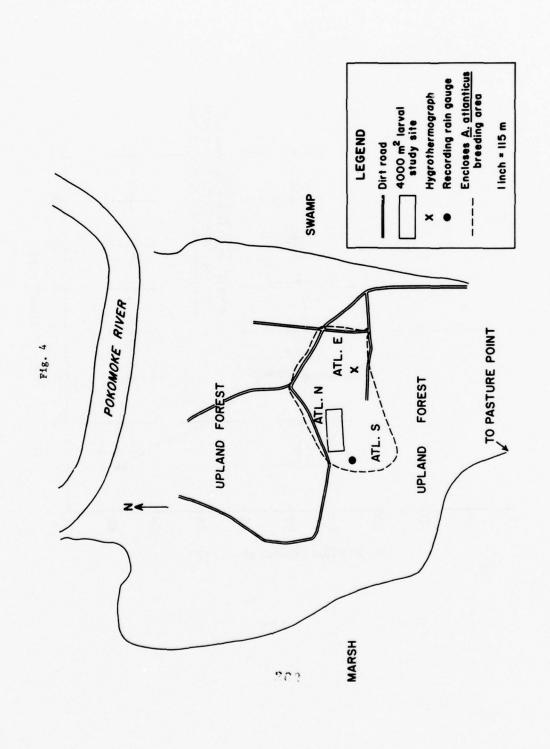
- Figure 14. A mesenchymal stylostome of <u>Leptotrombidium fletcheri</u> opening into the dermis of mouse ear tissue 24 hr. after attachment. a. central canal; b. chromophobic tube; c. eosinophilic coat. Hematoxylin and eosin stain.
- Figure 15. A mixed stylostome of <u>Leptotrombidium</u> arenicola 24 hr. after chigger attachment. a. epidermal portion; b. dermal portion. Hematoxylin and eosin stain.



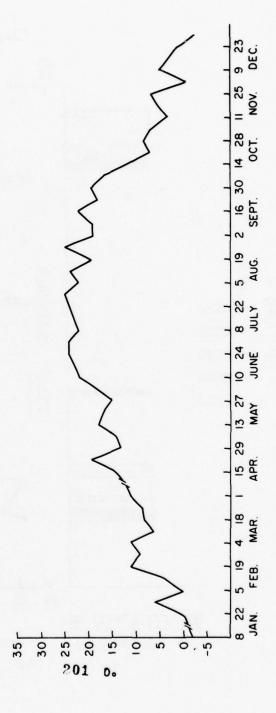
# PHOTOPERIOD AND TEMPERATURE REGIME

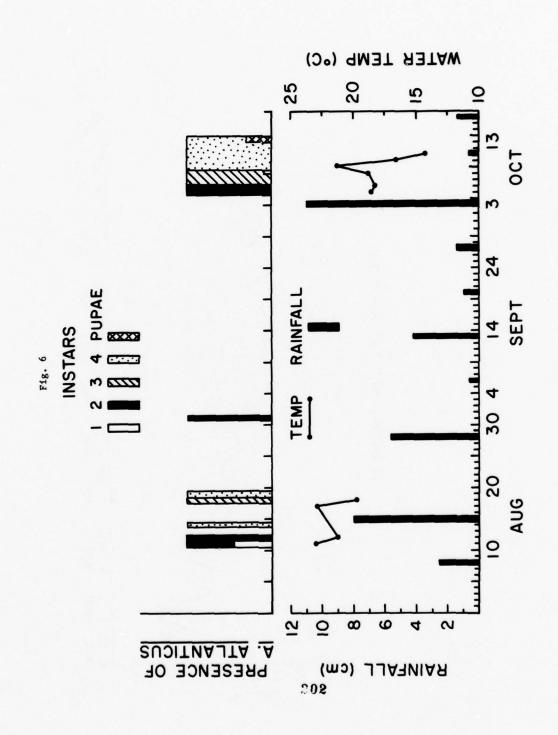


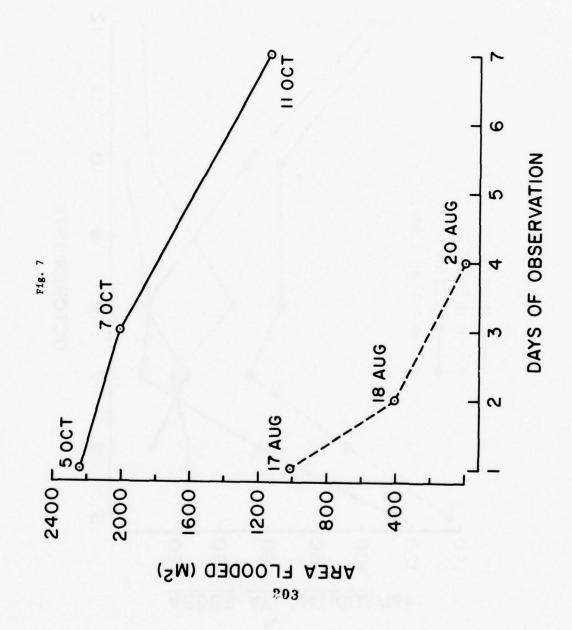


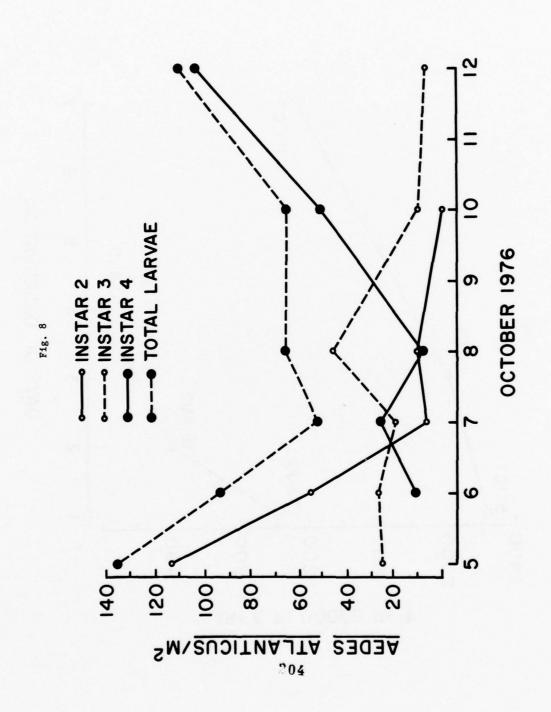


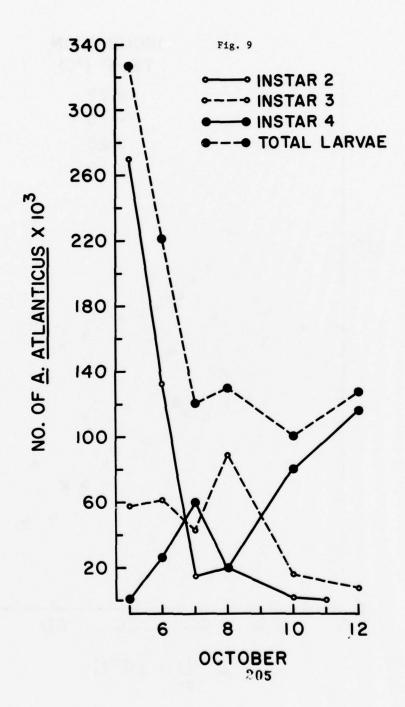
MEAN WEEKLY TEMPERATURE

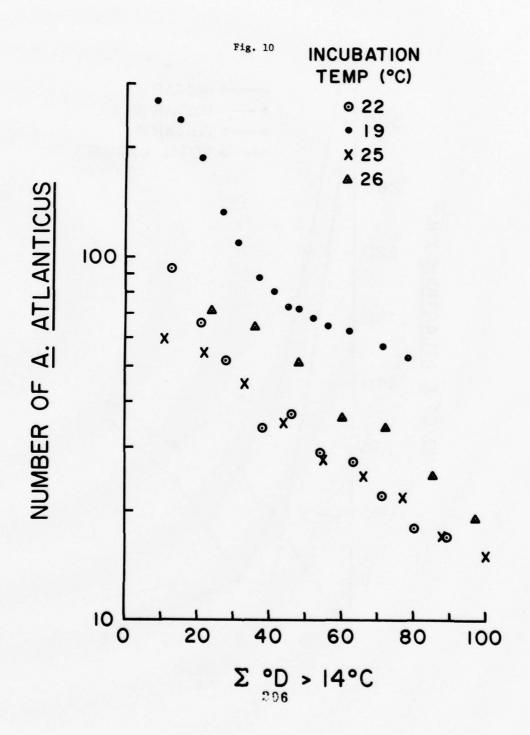




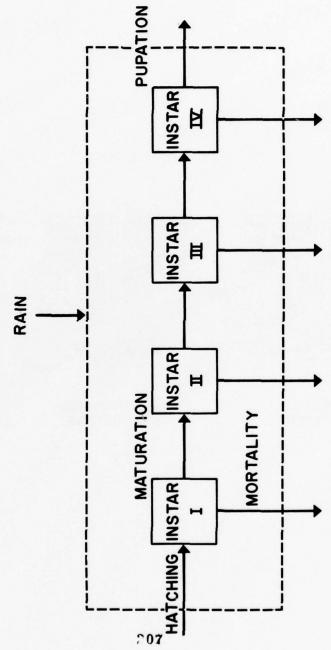








WATER TEMPERATURE



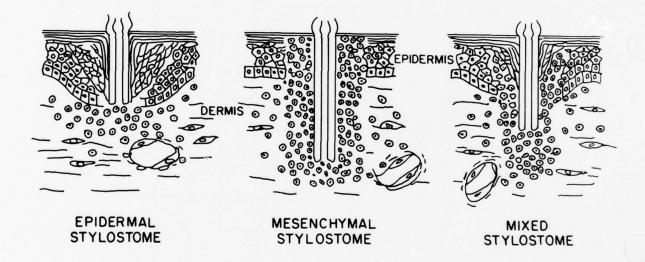
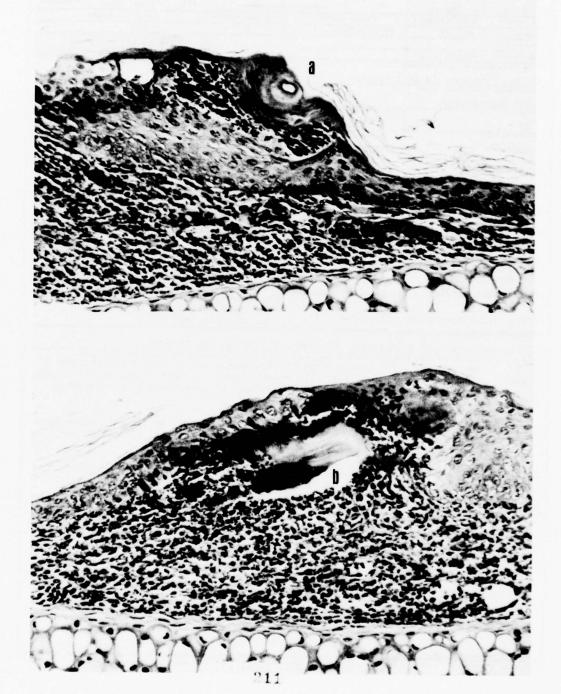






Fig. 15



	AND TECHNOLOG	Y WORK UNIT S	UMMARY		6448	77 10	-			RAE(AR)636
& DATE PREV SUMRY	4. KIND OF SUMMARY	S. SUMMARY SCTY	. WORK SECURITY	7. REGRAD			'N 1	SPECIFIC D	ATA-	S. LEVEL OF SUM
76 10 01	D. Change	U	U	NA NA		NL			140	A WORK WHET
10. NO./CODES:*	PROGRAM ELEMENT	PROJECT	NUMBER	TASK AR	EA NUMBER			WORK UNIT	NUMBER	
- PRIMARY	61102A	3M161102E	3501	1 0	0			122		
b. CONTRIBUTING										
c. CONTRIBUTING	CARDS 114F									
11. TITLE (Procede with	Security Classification Code	,•								
(U) Basic	Pharmacologic	al Studies								
12. SCIENTIFIC AND TE	CHHOLOGICAL AREAS									
012600 Pham	macology									
13. START DATE		14. ESTIMATED COM		IL FUNDIN	G AGENCY			16. PERFORMA		
68 07		CON	Γ	DA	1			C. In-	Hous	e
17. CONTRACT/GRANT					ACES ESTIM	ATE & PRO	E8510	NAL MAN YRS	& FUI	DE (In thousands)
A DATES/EFFECTIVE:	NA	EXPIRATION:								
b. NUMBER:*				FISCAL	77		3.	0		70
C TYPE:		& AMOUNT:		YEAR	UNNENT					
& KIND OF AWARD:		f. CUM. AMT.			78		3.	0		47
19. RESPONSIBLE DOD				M. PERFO	RMHG ORGA	NIZATION				
MAME: Walter	Reed Army Ins	titute of I	Research							Research
								hemistr	y	
ADDRESS: Wash	ington, DC 2	0012		ADDRESS:	Washi	ngton,	DC	20012		
				1						
								U.S. Academic I	q (lite tion	,
RESPONSIBLE INDIVIDU						R, Dr.		н.		
A CONTRACTOR OF THE CONTRACTOR										
HAME: RAPMUN						-576-3				
TELEPHONE: 202				SOCIAL S	ECURITY AC					
				SOCIAL S	ECURITY AC		ta:			
TELEPHONE: 202- 21. GENERAL USE	-576-3551	considered		SOCIAL S ASSOCIATE HAME:	ECURITY AC	CPT D.	W.,	Jr.		
TELEPHONE: 202- 21. GENERAL USE Foreign into	-576-3551 elligence not		d	SOCIAL S ASSOCIATE HAME:	ECURITY AC		W.,	Jr.		
TELEPHONE: 202: 21. GENERAL USE  Foreign into	-576-3551 elligence not	cation Code)		ASSOCIATE HAME:	CORTE,	CPT D.	W., B.			
TELEPHONE: 202: 21. GENERAL USE Foreign into	-576-3551 elligence not	cation Code)		ASSOCIATE HAME:	CORTE,	CPT D.	W., B.			
TELEPHONE: 202.  21. GENERAL USE  Foreign into  22. KEYBORDE (Proceda)  (U) Pharmac  23. TECHNICAL GBJECT	-576-3551 elligence not sacri old social closel ology; (U) Me	dicinals;	(U) Drugs;	ASSOCIATE HAME: HAME: S	CORTE, CCHUSTE	CPT D. R, MAJ	W., B.	y with Closelfice	of the code	ology of
TELEPHONE: 202:  31. GENERAL USE  Foreign into  32. KEVBORGE (Proceded)  (U) Pharmace  33. TECHNICAL GREET  23. (U) Resc	elligence not  ACR -18 10-218 Cloud  Ology: (U) Me  PUVE.* 24 APPROACH. 28  earch is dire	dicinals; PROGRESS (Fumion to cted toward	(U) Drugs;	ASSOCIATE HAME: NAME: S	CORTE, CHUSTE	CPT D.  R, MAJ  U) Tox  areas	W., B.	the pha	rmac	ology of
TELEPHONE: 202:  31. GENERAL USE  Foreign into  22. KEYBORGE (FRICA)  (U) Pharmac  33. TECHNICAL GBJECT  23. (U) Reso  potential di	-576-3551 elligence not sacri old social closel ology; (U) Me	dicinals;  PROGRESS (Pumish to cted toward ary importa	(U) Drugs; nd investiga ance, their	ASSOCIATE NAME: SHAME: S	CORTE, CCHUSTE lock; ( pecial	COUNT NUMB CORT D. R, MAJ U) Tox areas s, the	W., B.	the pha	rmac ns o	ology of f action,

- 24. (U) Drugs are tested in animal models specifically designed to pinpoint mechanisms of pharmacological effects, effects on physiological responses, and effects on protozoan systems. In vitro models are being used as well.
- 25. (U) 76 10 77 09 Both WR 149,024 and methylprednisolone protected dogs from a lethal dose of E. coli endotoxin when either drug was administered 45 minutes after the toxin. Further investigations on WR 2823 indicated a dose-related effect on duration of blood pressure response with a less pronounced dose relationship with heart rate in the cat. WR 2823 demonstrated a differential effect between digoxin toxicity and ouabain toxicity in the cat. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 76 30 Sep 77.

Project 3M161102BS01 BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 00 Biomedical Sciences

Work Unit 122 Basic pharmacological studies

Investigators.

Principal: Melvin H. Heiffer, Ph.D.

Associate: CPT D. Korte, MAJ B. Schuster, Dr. H. Lowensohn,

Dr. R. Rozman, SP4 M. Neidig

### 1. Description.

The basic research efforts of the department are directed towards several major areas. They are: the pharmacology of promising medicinal agents and of certain toxic substances; drug interactions with, and the nature of, adrenergic receptors; and the development of new or modification of existing techniques to characterize drug effects.

Appropriate pharmacological, physiological, electrophysiological and biochemical studies are conducted both <u>in vivo</u> and <u>in vitro</u>. Many of these studies emphasize interactions of potential drugs with standard pharmacological agents. An important feature is ready access to the vast inventory of serially related and diverse chemicals which can be used in detailed studies of the nature of drug interactions with biological systems.

## 2. Effects of WR 149,024 on lethal endotoxemia in the dog.

#### a. Background:

Gram-negative sepsis is still a major cause of shock despite advances in antibiotic therapy. Septic shock is characterized by inadequate tissue perfusion resulting from increased peripheral vascular resistance, pooling of blood in the microcirculation, diminished cardiac output and tissue anoxia. The vasoactive phenomena leading to this state of circulatory failure have been attributed to the release into the circulation of endotoxin, the lipopolysaccharide moiety of the gram-negative bacterial cell wall. Endotoxin is thought to exert its major effect in arterioles with alpha-adrenoreceptor innervation (Petersdorf, 1974). In experimental studies, this effect is observed as a prolonged and deleterious vasoconstriction accompanying a massive sympathetic discharge (Spink et al., 1966).

No single therapeutic agent has met with consistent success in the treatment of endotoxin shock. Since the shock state is accompanied by excessive stimulation of the alpha-adrenergic receptors, it is obvious that the sympathomimetic pressor agents so often used in other hypotensive states would be contraindicated, since they would further

reduce perfusion of vital organs (Petersdorf, 1974). Several pharmacological agents (isoproterenol, dopamine and corticosteroids) have had limited success in the treatment of septic shock. This success is most often associated with their prophylactic use or administration early in the course of the shock state. However, WR 149,024 (1,18-diamino-6,13-diaza-9,10-dithiaoctadecane tetrahydrochloride) has been shown to be effective in preventing mortality and modifying cardiovascular responses in a canine endotoxin shock model (Caldwell et al., 1975). This protective effect of WR 149,024 may be related to its reported production of vascular alpha-receptor blockade and enhancement of ascending aortic blood flow (Caldwell et al., 1972).

The studies with WR 149,024 (and most shock studies) involved treatment of the animal with the protective agent prior to the endotoxin insult. The present study was designed to determine the effectiveness of WR 149,024 when administered after the endotoxin insult. This regimen was selected to approximate more closely the conditions observed clinically with a patient already in septic shock. The effect of methylprednisolone treatment was also determined as a positive control.

## b. Materials and methods:

Thirty-six mongrel dogs were anesthetized with sodium pentobarbital (30 mg/kg i.v.). The right femoral artery and vein were catheterized for monitoring of arterial blood pressure and administration of drugs, respectively. Lead II electrocardiogram was also monitored. After stabilization of blood pressure and heart rate following surgery, E. coli endotoxin (Difco), 1.5 mg/kg, was administered intravenously over a 5 min period. The dogs were divided into 5 groups depending on their treatment. Ten dogs received no treatment after the endotoxin and served as controls. Seven dogs were given WR 149,024 (10 mg/kg of the salt, i.v.) 45 min post-endotoxin. An additional 10 dogs were given 10 mg/kg WR 149,024 60 min postendotoxin. Four dogs were given 30 mg/kg methylprednisolone sodium succinate (Solu-Medrol, Upjohn Co.) 45 min post-endotoxin. The final group of 5 dogs also was given 30 mg/kg methylprednisolone at 60 min post-endotoxin. The doses of WR 149,024 and methylprednisolone were dissolved in 5 ml of isotonic saline and administered slowly over a 5 min period. The dogs were monitored for 4 hr post-endotoxin administration, after which the catheters were removed, the vessels ligated and the wounds sutured. The dogs were then placed in the intensive care quarters where they received food and water ad libitum. Survivors were defined as those animals alive 72 hr after the endotoxin administration.

The rate of survival for the control groups was compared with the rate of survival in each of the treated groups using a

Chi-square 2 x 2 contingency table for each comparison (Shao, 1967). A computed Chi-square with a  $\underline{p}$  value equal to or less than 5% was considered an indication of a difference in survival rate attributable to the therapeutic regimen.

### c. Results:

The effects of WR 149,024 or methylprednisolone on survival of dogs given endotoxin are presented in Table 1. Chi-square analysis of these data indicated that both WR 149,024 and methylprednisolone increased survival rate when administered 45 min after endotoxin. When administered 60 min after endotoxin, the methylprednisolone produced a significant increase in survival rate while the increase in survival rate produced by WR 149,024 was not significant.

### d. Discussion:

These investigations indicated that WR 149,024 was effective when administered 45 min after the endotoxin insult. Previously, WR 149,024 pretreatment was shown to be beneficial in treatment of endotoxin shock (Caldwell et al., 1975). The present studies extending the efficacy of  $\overline{\text{WR}}$   $\overline{\text{149}}$ ,024 to 45 min after administration of the endotoxin are most promising since this model more closely approximates the conditions of the patient already in septic shock.

The beneficial effects of the WR 149,024 treatment are essentially lost when administered 60 min after the endotoxin insult. Therefore, changes in the shock model must occur between 45 and 60 min post-endotoxin administration which limit the effectiveness of WR 149,024. However, methylprednisolone, a synthetic glucocorticoid preparation, was still effective at 60 min. Methylprednisolone and other steroids are thought to produce their protective effects in shock state by a membrane-stabilizing action which reduces lysosomal enzyme release (Glenn and Lefer, 1971). This would tend to reduce the probability that WR 149,024 is effective via the membrane-stabilization mechanisms associated with steroid therapy. Additional studies with this shock model characterizing the hemodynamic, metabolic and subcellular processes occurring between 45 and 60 min after administration may help define the protective action of WR 149,024.

WR 149,024 is a promising agent for use in shock states. The relative ineffectiveness of WR 149,024 versus steroid at 60 min postendotoxin should not detract from its potential use. Massive steroid therapy produces many side-effects which complicate the long term prognosis. WR 149,024 has been reported to produce no abnormalities on clinical examination, ophthalmoscopy, gross necropsy or histology when administered at dosages of up to 20 mg/kg for 14 days to dogs and monkeys (WR 149,024 IND, Tabs J and K). Thus, WR 149,024 appears

promising as an effective anti-shock compound without the toxic potential of currently used therapeutic compounds.

## 3. Characterization of the initial cardiovascular actions of WR 2823.

## a. Background:

In 1969, Heiffer and co-workers first described the pharmacology of an aliphatic sulfur-containing compound (Heiffer et al., 1969). This compound, S-2-[5-(aminopentyl)amino]ethylphosphorothioic acid or WR 2823, was shown to have both immediate and prolonged cardiovascular activity. When WR 2823 was administered intravenously it produced an immediate hypotension and bradycardia in several animal species. These initial responses are followed by a longlasting, selective and specific alpha-adrenergic blockade (Heiffer et al., 1969; Herman et al., 1971). WR 2823, perhaps because of its alpha-adrenergic blocking action, has considerable efficacy in treatment of endotoxin and hemorrhagic shock in different animal models (Vick et al., 1969; Vick and Heiffer, 1970; Vick et al., 1973). However, the initial cardiodepressor responses could limit the usefulness of WR 2823 or its congeners in the treatment of shock states in man. Therefore, we have attempted to describe in detail the nature of these initial cardiovascular responses.

Previously we reported that neither atropine, lysergic acid diethylamide, nor diphenhydramine altered the initial responses of WR 2823. These data eliminated stimulation of the Bezold-Jarish reflex, release of 5-hydroxytryptamine, and release of histamine, respectively as mechanisms of action of the initial hypotension and bradycardia observed on administration of WR 2823. Pretreatment with the beta-adrenergic blocking agent, propranolol, or the alphaadrenergic blocking agent, phenoxybenzamine, attenuated the bradycardia but not the hypotension produced initially by WR 2823. Reserpinization, which depletes the sympathetic nervous system of its neurotransmitter, norepinephrine, attenuated both the hypotension and bradycardia produced by WR 2823. Ganglionic blocking agents such as mecamylamine and hexamethonium, which interrupt autonomic transmission, abolished the initial cardiovascular responses of WR 2823. These data indicated that WR 2823 was interacting with the autonomic nervous system, probably at central sites. To confirm this hypothesis, animals were transected between the first and second cervical vertebrae or decerebrated at the intercollicular level and WR 2823 was administered. Spinal cord transection abolished the initial cardiovascular responses while decerebration had no effect. This implicates the lower brain stem as the possible site of action for the initial cardiovascular effects of WR 2823.

Our present studies followed two lines of investigation. The first was to determine whether a dose-response relationship exists for the initial cardiovascular responses to WR 2823 administration. Our studies have shown that 25 mg/kg and 50 mg/kg of WR 2823 had similar effects. If there appears to be a threshold dose for these effects, multiple administrations of subthreshold doses may then decrease the potentially adverse effects initially observed with WR 2823 administration. The second course of investigation was to explore the possible mechanism of the initial response. Clonidine, an antihypertensive agent, has been shown to produce bradycardia and hypotension by direct action, stimulating alpha-adrenergic receptors located in medullary vasomotor centers (Tangri et al., 1977). Investigations were begun to determine whether pretreatment with clonidine potentiated or attenuated the initial cardiovascular responses of WR 2823 in an attempt to clarify further the mechanism responsible for the WR 2823 response.

### b. Materials and methods:

Cats weighing 2.5 to 3.5 kg were anesthetized with pento-barbital sodium, 40 mg/kg ip. The right femoral vein was cannulated for drug injection. Cannulae were also inserted into the right femoral artery and trachea for monitoring blood pressure and respiration. Needle-tipped electrodes were inserted into the appropriate limbs to record Lead II of the electrocardiogram. Heart rate was determined from the interval of the R wave by means of a cardiotach preamplifier. Respiration, Lead II electrocardiogram, arterial pressure, mean arterial pressure and heart rate were recorded on a Hewlett-Packard polygraph.

Following a 30 min equilibration period, 12.5 mg/kg (4 cats), 6.25 mg/kg (4 cats) or 3.125 mg/kg (3 cats) doses of WR 2823, dissolved in 10 ml of isotonic saline, were injected slowly over a 4 min period. The various measurements were monitored for 60 min after the WR 2823 injection and the experiment was then terminated.

In a second series of experiments, 3 cats were pretreated with clonidine HCl, 0.1 mg/kg, injected over a 2 min period. The animals were monitored for 30 min before WR 2823 administration. WR 2823, 50 mg/kg, was injected slowly over 4 min. The various measurements were monitored for 60 min and the experiment was then terminated.

Control observations of the various physiological measurements were made just prior to pharmacological pretreatment, if any, and before WR 2823 administration. The percentage change from the appropriate control value was determined at 1, 5, 15, 30 and 60 min after injection of WR 2823.

### c. Results:

The dose responses of WR 2823 on mean arterial pressure and heart rate are given in Tables 2 and 3, respectively. Previously obtained data for 50 mg/kg and 25 mg/kg dosages of WR 2823 are included in these tables for comparison purposes. Inspection of the mean arterial pressure data indicate the magnitude of the vaso-pressor response was equivalent over a dose range from 6.25 mg/kg to 50 mg/kg WR 2823. However, the duration of this response seemed to decrease with decreasing doses. With the 50 mg/kg dose the blood pressure had returned to 72% of control levels within 60 min. The blood pressure following the 25 mg/kg and 12.5 mg/kg dosages had returned to 89% and 88% of control values within 15 min. After WR 2823, 6.25 mg/kg, the blood pressure returned to 93% of control levels within 5 min. WR 2823, 3.125 mg/kg, had only a minimal effect on blood pressure at one min post-injection.

The effects of different doses of WR 2823 on heart rate were not as definite as the hypotensive responses. There was no dose-response pattern to the maximum decrease in heart rate observed at doses ranging from 6.25 mg/kg to 50 mg/kg. The duration of the bradycardia was generally shorter with decreasing doses of WR 2823. With the highest dose of WR 2823, heart rates recovered within 60 min to control levels. After doses of 25 mg/kg to 6.25 mg/kg the heart rates recovered to 90% of control within 30 min. WR 2823, 3.125 mg/kg, produced only minimal changes in the heart rate.

The effects of pretreatment with clonidine on the initial blood pressure and heart rate responses of 50 mg/kg WR 2823 are given in Table 4. Pretreatment with 0.1 mg/kg clonidine produced a decrease to 56% of control in heart rate and to 82% of control in mean arterial pressure 30 min post-injection. Clonidine pretreatment accentuated the initial hypotension and eliminated the initial bradycardia produced by 50 mg/kg WR 2823.

#### d. Discussion:

Intravenous administration of WR 2823 consistently induced hypotension and bradycardia at doses ranging from 6.25 to 50.0 mg/kg while 3.125 mg/kg had minimal effects on heart rate and blood pressure. There appeared to be no dose-response relationship in the magnitude of the hypotension or bradycardia. This was consistent with data obtained with WR 149,024. WR 149,024, which is a dimer of dephosphorylated WR 2823, also produced hypotension and bradycardia over a dose range of 6.25 to 25.0 mg/kg in the anesthetized dog (Caldwell et al., 1972). As with WR 2823, the 6.25 mg/kg dose of

WR 149,024 depressed blood pressure and heart rate to the same extent as the 25 mg/kg dose. Thus, the magnitude of the initial cardiovascular effects was not dose dependent.

The duration of the initial hypotensive response of WR 2823 appeared to be dose-dependent in nature. This was especially true with the smaller doses. However, the response to larger doses was more complex. WR 2823 reversed the pressor response to epinephrine at dosages greater than 25 mg/kg in the rat (Herman et al., 1971). This effect became apparent within 15 to 30 min and was maximal by 60 min. The failure of the blood pressure in cats administered 50 mg/kg of WR 2823 to return to control values after 60 min was probably a function of the developing alpha-adrenergic blockade. A dose-dependent relationship of the duration of the initial heart rate response to WR 2823 administration was not as apparent as with the hypotension. This was probably a function of a reflexive increase in heart rate (in response to the initial hypotension produced by WR 2823) superimposed on the initial bradycardia.

The initial effects of WR 2823 were very similar to the reported actions of clonidine in that both agents produce a hypotension and bradycardia. The hypotension and bradycardia produced by clonidine were attributed to alpha-adrenergic stimulation in the vasomotor center of the medulla (Tangri et al., 1977). We have shown previously that the initial hypotension and bradycardia produced by WR 2823 resulted from an interaction with vasomotor centers in the brain stem. Therefore, pretreatment with clonidine may alter the initial responses of WR 2823. If clonidine and WR 2823 acted via similar mechanisms an enhanced hypotension and bradycardia would be expected. If the initial effects of WR 2823 were due to alpha-adrenergic blockade of vasomotor centers then WR 2823 would be expected to antagonize the clonidine-induced hypotension and bradycardia.

The results obtained when WR 2823 was administered after clonidine pretreatment were only partially predicted. WR 2823 did enhance the hypotensive response to clonidine but did not augment the clonidine-induced bradycardia. Clonidine produces hypotension by activation of central mechanisms to reduce sympathetic tone while the bradycardia is due to both a centrally produced reduction in sympathetic tone and an enhanced vagal tone due to activation of peripheral baroreceptors (Tangri et al., 1977). The additional hypotension observed after WR 2823 in animals pretreated with clonidine may be due to a further reduction in sympathetic tone. The decrease in sympathetic tone would also decrease the heart rate. However, this additional vasopressor response may depress the baroreceptor reflex originally stimulated by clonidine. This reduction in baroreceptor firing could negate any action of WR 2823 to

decrease heart rate by a reduction in sympathetic tone. Early studies which indicated that atropine did not attenuate the initial bradycardia produced by WR 2823 are consistent with this hypothesis that the initial effects of WR 2823 are due to central inhibition of sympathetic tone.

# 4. Antiarrhythmogenic potential of WR 2823.

### a. Background:

WR 2823, an aliphatic sulfur-containing compound, has both immediate and prolonged cardiovascular activity. This activity consists of an immediate hypotension and bradycardia followed by a long-lasting selective alpha-adrenergic blockade (Heiffer et al., 1969). WR 2823 has been reported to decrease mortality from hemorrhage in animals (Vick et al., 1973). Hemorrhage has been reported to produce subendocardial lesions which, by disrupting conduction, may produce arrhythmias that result in a decrease in cardiac output which would augment the positive feedback cycle leading to irreversible shock (Hackel et al., 1974). Thus, prevention of arrhythmias may be one aspect of the protective property of WR 2823 in shock states. To determine whether a possible antiarrhythmic action could contribute to its protective properties in shock states, WR 2823 was evaluated for its antiarrhythmic potential against both ouabain- and digoxin-induced toxicity.

## b. Materials and methods:

Adult cats of either sex (2.2 - 3.8 kg) were anesthetized with sodium pentobarbital (40 mg/kg, ip). The trachea was cannulated with an endotracheal tube for monitoring respiration. The left femoral artery was cannulated for recording arterial pressure. Lead II of the electrocardiogram was recorded for measuring heart rate and detecting changes in cardiac rhythm. The right femoral vein was cannulated for glycoside infusions and the left femoral vein was cannulated for other drug injections. Lead II of the electrocardiogram, arterial pressure, heart rate and respiration were recorded on a Hewlett-Packard Model 7828A recorder. Lead II of the electrocardiogram was also displayed on the visoscope component of the recorder for continuous monitoring of rhythm changes. Rectal temperature was measured with a Yellow Springs telethermometer and maintained at 37°C by a heating pad.

The cats were divided into 3 groups depending on the glycoside used and the rate of infusion of the glycoside. The first group consisted of those animals given digoxin (Lanoxin, Burroughs-Wellcome, Inc.) at an infusion rate of 2  $\mu g/kg/min$ . The second group consisted of those animals given ouabain (Calbiochem) at an

infusion rate of 2  $\mu g/kg/min$  . The third group consisted of an additional group of animals given ouabain but at an infusion rate of 1.25  $\mu g/kg/min$  .

The animals in the first group were divided into 5 subgroups. The first subgroup consisted of those animals infused with digoxin without drug pretreatment. The second subgroup consisted of those animals given WR 2823 Lot AE (50 mg/kg i.v.) slowly over a 5 min period followed one hr after the start of the WR 2823 injection by the digoxin infusion. The third subgroup consisted of those animals given a lower dose of WR 2823 (35 mg/kg i.v.) followed one hr later by the digoxin infusion. The fourth subgroup consisted of those animals given propranolol HCl (Inderal, Ayerest Company), 2 mg/kg i.v. slowly over a 5 min period followed by the digoxin infusion 15 min after the start of the propranolol injection. The fifth subgroup consisted of those animals given phenoxybenzamine, 7.5 mg i.v. (Dibenzyline, Smith, Kline and French Labs) slowly over a 5 min period beginning 45 min prior to the digoxin infusion.

The second group of cats was divided into 4 subgroups. The animals in the first subgroup received no drug pretreatment. The second subgroup received WR 2823, 50 mg/kg i.v., the third subgroup received propranolol HCl, 2 mg/kg i.v., and the fourth subgroup received phenoxybenzamine HCl, 7.5 mg/kg i.v., all prior to a 2  $\mu g/kg/min$  ouabain infusion. The time sequence of the pretreatments in this group was the same as that described for the first group given digoxin.

The third group of cats was divided into 3 subgroups. The first subgroup received no drug pretreatment, the second group was pretreated with WR 2823, 50 mg/kg i.v., and the third subgroup received propranolol HCl, 2 mg/kg i.v., all prior to a 1.25  $\mu g/kg/$  min ouabain infusion. The time sequence of these pretreatments was the same as described for groups I and II.

Both doses of WR 2823 and the propranolol were dissolved in 5 ml of isotonic saline. The phenoxybenzamine was dissolved in 0.2 ml of glacial acetic acid and diluted to 5 ml total volume with isotonic saline. The ouabain was dissolved in and the digoxin was diluted with isotonic saline so that the appropriate dose was administered at a rate of 0.181 ml/min.

The toxic dose and lethal dose of the two glycosides were calculated from the infusion time. The toxic dose was considered to be the dose of glycoside that produced 3 consecutive contractions of ventricular origin. The lethal dose was considered to be the dose of glycoside that produced ventricular fibrillation. The doses necessary to produce the toxic and lethal endpoints after the pretreatments were compared with the control endpoints using Dunnett's

multiple comparison procedure (Zar, 1974) with a  $\underline{p}$  value of 0.05 or less considered significant.

### c. Results and discussion:

The results of WR 2823, propranolol or phenoxybenzamine pretreatment on digoxin toxicity are given in Table 5. The dose of digoxin that produced ventricular tachycardia was  $126.6\pm7.9~\mu g/kg$ . Both 50 mg/kg WR 2823 (36.4%) and propranolol (58%) produced significant increases in the dose of digoxin necessary to produce ventricular tachycardia. However, neither the lower dose of WR 2823 (26.9%) nor phenoxybenzamine (20%) produced increases that were significantly greater than the control values. The fibrillation dose of digoxin in control cats was  $158.8\pm6.3~\mu g/kg$ . In addition to 50 mg/kg WR 2823 (30.5%) and propranolol (50.2%), 35 mg/kg WR 2823 (25.7%) also produced a significant increase in the lethal dose of digoxin. Furthermore, the protective effect of WR 2823 appears to be related to the dose administered. Phenoxybenzamine had little effect (9.6%) on the lethal dose of digoxin.

The results of pretreatment with WR 2823, propranolol or phenoxybenzamine on ouabain toxicity when infused at a rate of 2  $\mu$ g/kg/min are given in Table 6. Ouabain-induced ventricular tachycardia was observed after infusion of 68.6  $\pm$  3.6  $\mu$ g/kg in control animals. Pretreatment with 50 mg/kg WR 2823, propranolol or phenoxybenzamine did not significantly increase the dose of ouabain necessary to produce ventricular tachycardia. But propranolol did increase the dose necessary to produce ventricular tachycardia by 15.1  $\mu$ g/kg. The fibrillatory dose of ouabain in the control animals was 97.2  $\pm$  6.0  $\mu$ g/kg. WR 2823, 50 mg/kg, produced a slight decrease and phenoxybenzamine a slight increase in the toxic dose of ouabain. Propranolol produced a significant increase of 26.9% in the lethal dose of ouabain.

Since ouabain was more potent than digoxin in producing toxicity, the rate of ouabain infusion was lowered to 1.25  $\mu g/kg$  in the third group of cats. The results of pretreatment with 50 mg/kg WR 2823 and propranolol in this slower infusion rate of ouabain are given in Table 7. The dose of ouabain necessary to produce ventricular tachycardias at the lower infusion rate was 71.5  $\pm$  7.5  $\mu g/kg$ . The lethal dose of ouabain given at the slower infusion rate was 84.9  $\pm$  6.4  $\mu g/kg$ . These doses are no different than the higher infusion rates but the time frame of toxicity more closely resembled the time of onset previously determined for digoxin. At the slower infusion rate, propranolol produced significant increases in both the dose for ventricular tachycardia and fibrillation. Once again 50 mg/kg WR 2823 had little effect on ouabain toxicity.

The action of WR 2823 against digitalis-induced arrhythmias was investigated in order to determine whether an antiarrhythmic effect may, in part, be responsible for the effectiveness of WR 2823 in treatment of shock states. Phentolamine and phenoxybenzamine, alpha-adrenoreceptor blocking agents, have been shown to be effective against ouabain-induced arrhythmias (Ettinger et al., 1969) and digoxin-induced arrhythmias (Rothaus and Powell,  $\overline{1975}$ ), respectively. Since WR 2823 has alpha-adrenoreceptor blocking activity, a similar antiarrhythmic spectrum was anticipated. Analysis of the data indicated that WR 2823 was effective against digoxin toxicity in a dose-related manner. Rothaus and Powell (1975) suggested that phenoxybenzamine was effective against digoxininduced arrhythmias because it antagonized the alpha-mediated sympathetic constriction of coronary arteries produced by digoxin. However, phenoxybenzamine was not effective in delaying the onset of ouabain or digoxin toxicity in this study. Furthermore, WR 2823 was not effective against ouabain toxicity in this experimental model. This indicates that the efficacy of WR 2823 in delaying the onset of digoxin toxicity in the cat is not attributable to its alpha-receptor blocking activity.

The model used to assess digitalis toxicity is very important since even choice of anesthesia has been shown to affect ouabain toxicity (Stickney, 1974). Ouabain, when infused at equal rates, produced toxic effects at lower doses than digoxin; thus, this ouabain model may have been too toxic to show a protective action by WR 2823. Therefore, a lower infusion rate of ouabain was studied. This infusion rate was designed to approximate the time course of digoxin toxicity. Once again, WR 2823 was not effective in delaying ouabain toxicity. These findings were inconclusive in establishing an antiarrhythmic effect as a mechanism for the protective action of WR 2823 in shock states. However, these results do suggest a difference in mechanism for the toxicity produced by ouabain and digoxin.

The results of these studies are interesting since only recently has differentiation of the toxicity produced by cardiac glycosides been reported. Kelliher and Roberts (1974) suggested that beta-adrenoreceptor blocking agents protected against digitalis toxicity by different mechanisms depending on the glycoside used. Digoxin-induced arrhythmias could be prevented by beta-blocking doses of these agents, which were ineffective against ouabain-induced arrhythmias. Only doses of the beta-blocking agents which produce neural depression were effective against ouabain-induced arrhythmias. The present study demonstrates that the alpha-blocking agent, WR 2823, is effective against digoxin-induced toxicity but not ouabain-induced toxicity. Thus, the varying efficacy of WR 2823 in preventing digitalis toxicity may be a function of inherent differences in the toxicity of the cardiac glycosides.

Digitalis glycosides have both a direct toxic action in the heart and an indirect effect mediated via an interaction with the sympathetic nervous system. Recent studies with transplanted and denervated hearts indicate that the indirect neural aspects of digitalis glycosides may be a more important component of their toxicity than the direct effects (Gillis et al., 1975). The indirect neural effect associated most often with digitalis toxicity has been sympathetic stimulation (Gillis et al., 1975). However, the site(s) of digitalis interaction within the sympathetic nervous system has not been defined. Recent evidence indicates that the primary site of digoxin interaction is peripheral rather than central (Weaver et al., 1976) while ouabain has little effect on peripheral reflexes (McRitchie and Vatner, 1976) but has profound effects on the posterior hypothalamus to produce sympathetic stimulation (Saxena and Bhargava, 1975). In addition, Dutta and Marks (1966) indicate that digoxin is concentrated to a greater extent in the adrenal gland than is ouabain. Furthermore, 6-hydroxydopamine, an agent that depletes 95% of the catecholamines in the heart but only 35% of the catecholamines in the adrenal gland protects against ouabain toxicity but not digoxin toxicity (Roberts et al., 1976). Thus, it appears that ouabain may have more of an effect centrally to produce an increase in sympathetic tone while digoxin may act through the adrenal gland directly, as well as through other peripheral components, to produce an increase in sympathetic tone. The observation that WR 2823 is not effective against ouabain toxicity may mean that WR 2823 does not cross the blood-brain barrier in sufficient quantities to produce neural depression or that WR 2823 lacks appreciable neural depressant activity.

Recent studies indicate that digitalis toxicity is due to Na+, K+-ATPase inhibition (Beeler, 1977). Agents such as N-ethyl-maleimide and p-chloromercuribenzene sulfonate that are able to interact with sulfhydral groups of the Na+, K+-ATPase are also inhibitory (Brody and Akera, 1977). WR 2823, a sulfur-containing aliphatic amine, is thought to produce its alpha-blocking effect by combining with sulfhydral groups on the alpha receptor (Herman et al., 1971). WR 2823 could also combine with sulfhydral groups of the Na+, K+-ATPase. Thus, WR 2823 could prevent glycoside binding to the Na+, K+-ATPase molecule at glycoside concentrations which are normally inhibitive. This action of WR 2823 would be more apparent in delaying digoxin toxicity since digoxin toxicity is not primarily related to central mechanisms.

Table 1

Effect of WR 149,024 or Methylprednisolone on Survival of Dogs Given Endotoxin

Treatment	=	# of 72 hr Survivors	# of Deaths	Calculated	Tabular <sup>a</sup>	Significant at 5% Level
	1					
Control	10	က	7	•	•	1
WR 149,024 (10 mg/kg) 45 min	7	9	-	5.10	3.84	Yes
Methylprednisolone (10 mg/kg) 45 min	4	4	0	5.60	3.84	Yes
WR 149,024 (10 mg/kg) 60 min	10	Ŋ	.c	0.50	3.84	No
Methylprednisolone (10 mg/kg) 60 min	2	ıs	0	6.54	3.84	Yes

 $^{\mathrm{a}}$ Tabular  $_{\mathrm{x}}{}^{\mathrm{2}}$  for a 5% level of significance and 1 degree of freedom.

Table 2 Dose Response of WR 2823 on Mean Arterial Pressure

		3		Б	
Minutes		52	% of Control Values Dose of WR 2823	3	
WR 2823	50 mg/kg	25 mg/kg	12.5 mg/kg	6.25 mg/kg	3.125 mg/kg
-	9 + 29	51 + 2	62 + 6	64 + 6	92 + 3
S	66 + 4	64 + 4	74 + 8	93 + 6	98 + 2
15	2 + 69	6 + 68	88 + 2	95 + 5	100 + 0
30	68 + 5	114 + 15	102 ± 3	9 + 66	99 + 1
09	72 ± 4	117 ± 16	102 ± 2	9 + 66	99 + 1
# of Expt	7	8	4	4	8
Control Values (mmHg)	128 + 12	97 + 12	99 + 14	123 ± 23	132 ± 14

a Values are mean ± SEM.

Table 3 Dose Response of WR 2823 on Heart Rate

		%	% of Control Values <sup>a</sup>	Sa	
Minutes After			Dose of WR 2823	3	
WR 2823	50 mg/kg	25 mg/kg	12.5 mg/kg	6.25 mg/kg	3.125 mg/kg
-	87 ± 10	85 + 1	73 ± 3	77 ± 3	93 ± 2
2	81 + 7	83 + 1	71 ± 3	87 ± 2	94 + 1
15	77 + 77	104 ± 15	76 ± 3	97 + 2	97 ± 1
30	88 + 7	111 + 111	91 + 4	101 ± 2	99 + 1
09	105 ± 7	118 ± 14	94 + 3	104 ± 2	100 + 0
# of Expt	7	8	4	4	က
Control Values (bpm)	176 ± 9	159 ± 9	178 ± 18	91 + 961	208 ± 16

<sup>a</sup>Values are mean <u>+</u> SEM.

Table 4

Effect of Pretreatment with Clonidine 0.1 mg/kg on Mean Arterial

Pressure and Heart Rate Responses Following a Four Minute Intravenous

Infusion of WR 2823 (50 mg/kg) in the Anesthetized Cat

	% of C	Control Values <sup>a</sup>
	Heart Rate	Mean Arterial Pressure
After Clonidine	56 <u>+</u> 6	82 <u>+</u> 1
After WR 2823 <sup>b</sup> (min)		
1	111 <u>+</u> 6	44 <u>+</u> 5
5	109 <u>+</u> 6	52 <u>+</u> 5
15	101 <u>+</u> 2	65 <u>+</u> 10
30	100 <u>+</u> 2	71 <u>+</u> 8
60	96 <u>+</u> 2	84 <u>+</u> 4
Pre-Clonidine Values	182 <u>+</u> 25 bpm	122 <u>+</u> 20 mmHg

 $<sup>^{\</sup>rm a}$  Values are mean  $\pm$  SEM for 3 cats.

 $<sup>^{\</sup>mathrm{b}}$ Control represents post-clonidine values.

Table 5

Effect of WR 2823, Propranolol and Phenoxybenzamine on Digoxin Toxicity

		IV	<b>و</b> -	VFª	à
Pretreatment Drug (Dose)	اء	Dose (µg/kg) % of Control	% of Control	Dose (µg/kg) % of Control	% of Control
Control	9	$126.7 \pm 7.9^{C}$	100.0	158.5 ± 6.3	100.0
WR 2823 (50 mg/kg)	9	172.2 ± 18.4 <sup>d</sup>	136.4	$207.3 \pm 15.6^{d}$	130.5
WR 2823 (35 mg/kg)	2	160.8 ± 19.2	126.9	199.6 ± 20.9 <sup>d</sup>	125.7
Propranolol (2 mg/kg)	9	200.0 ± 8.6 <sup>d</sup>	158.0	238.5 ± 9.1 <sup>d</sup>	150.2
Phenoxybenzamine (7.5 mg/kg)	4	4 152.0 ± 12.3	120.0	174.0 ± 15.8	109.6

 $^{\mathrm{a}}\mathrm{VI}$  - dose of digoxin infused when ventricular tachycardia occurred.

 $^{\mathsf{b}_{\mathsf{VF}}}$  - dose of digoxin infused when ventricular fibrillation occurred.

CValues are mean ± SEM.

<sup>d</sup>Significantly different (p <0.05) from control, Dunnett's Multiple Comparison Procedure.

Table 6

Effect of WR 2823, Propranolol and Phenoxybenzamine on Ouabain Toxicity

		TV V	-a	VF	P
Pretreatment Drug (Dose)	<b>=</b>	Dose (µg/kg)	% of Control	Dose (µg/kg)	% of Control
Control	9	68.7 ± 3.6 <sup>c</sup>	100.0	$97.2 \pm 6.0$	100.0
WR 2823 (50 mg/kg)	9	$69.7 \pm 4.0$	9.101	89.8 + 4.3	92.4
Propranolol (2 mg/kg)	9	83.7 ± 4.9	1.9.1	123.3 ± 8.4 <sup>d</sup>	126.9
Phenoxybenzamine (7.5 mg/kg)	4	75.0 ± 7.7	109.2	101.0 ± 11.9	103.9

 $^{a}$ VT - dose of ouabain infused (2  $_{
m \mu}g/kg/min)$  when ventricular tachycardia occurred.

 $^{b}$ VF - dose of ouabain infused (2  $_{\mu}g/kg/min$ ) when ventricular fibrillation occurred.

CValues are mean ± SEM.

dsignificantly different (p <0.05) from control, Dunnett's Multiple Comparison Procedure.

Table 7

Effect of WR 2823 and Propranolol on Toxicity Produced by a Slow Ouabain Infusion

		VT		VF	9
Pretreatment Drug (Dose)	<b>=</b> 1	Dose (µg/kg)	% of Control	Dose (µg/kg)	% of Control
Control	2	71.5 ± 7.5°	100.0	$84.9 \pm 6.4$	100.0
WR 2823 (50 mg/kg)	2	78.5 ± 5.2	109.8	94.3 ± 8.6	1.111
Propranolol (2 mg/kg)	2	101.3 ± 9.9 <sup>d</sup>	141.7	118.5 ± 8.8 <sup>d</sup>	139.6

 $^{b}$ VF - dose of ouabain infused (1.25  $\mu g/kg/min)$  when ventricular fibrillation occurred.  $^{a}VT$  - dose of ouabain infused (1.25  $_{\mu}g/kg/min)$  when ventricular tachycardia occurred. CValues are mean ± SEM. dsignificantly different (p <0.05) from control, Dunnett's Multiple Comparison Procedure.

Project 3M161102BS01 BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 00 Biomedical Sciences

Work Unit 122 Basic pharmacological studies

### Literature Cited.

### References:

- 1. Petersdorf, R.G.: Septic shock, in <u>Harrison's Principles of Internal Medicine</u>. Vol. 1, 7th Ed., pp 734-739, 1974.
- 2. Spink, W.W., Reddin, J., Zak, S.S., Peterson, M., Starzeck, B. and Seljeskog, E.: Correlation of plasma catecholamine levels with hemodynamic changes in canine endotoxin shock. J. Clin. Invest. 45:78-85, 1966.
- 3. Caldwell, R.W., Vick, J.A. and Heiffer, M.H.: Modification of experimental endotoxin shock with WR 149,024. Circ. Shock  $\underline{2}$ : 265-275, 1975.
- 4. Caldwell, R.W., Heiffer, M.H. and Herman, E.H.: The cardio-vascular actions of WR 149,024 (1,18-diamino-6,13-diaza-9,10-dithiaoctadecane tetrahydrochloride). Brit. J. Pharmacol. 46:637-646, 1972.
- 5. Shao, S.P.: <u>Statistics for Business and Economics</u>. Charles E. Merrill Books, Inc., Columbus, Ohio, 1967.
- 6. Glenn, T.M. and Lefer, A.M.: Antitoxic effects of methyl-prednisolone in hemorrhagic shock. Eur. J. Pharmacol. 13:230-238, 1971.
- 7. Heiffer, M.H., Herman, E.H., Vick, J.A., Demaree, G.D., Mundy, R.L., Reynolds, D.G. and Jacobus, D.P.: On the <u>alpha-adrenergic blocking properties of WR 2823</u>. Fed. Proc. 28:611, 1969.
- 8. Herman, E., Heiffer, M., Demaree, G. and Vick, J.: The <u>alpha-adrenergic blocking properties of a series of straight chain</u> sulfur containing compounds. Arch. int. Pharmacodyn. 193:102-110, 1971.
- 9. Vick, J.A., Heiffer, M.H. and Jacobus, D.: Treatment of hemorrhagic shock with WR 2823. Physiol. 12:383, 1969.
- 10. Vick, J.A. and Heiffer, M.H.: Prevention of endotoxin shock with a new <u>alpha-adrenergic blocking agent</u>. Pharmacologist <u>12</u>:284, 1970.

- 11. Vick, J.A., Heiffer, M.H., Roberts, C.R., Caldwell, R.W. and Nies, A.: Treatment of hemorrhagic shock with a new vasodilator. Mil. Med. 138:490-494, 1973.
- 12. Tangri, K.K., Petty, M., Wing, L.M.H. and Reid, J.L.: Mechanism of cardiovascular effects of clonidine in conscious and anesthetized rabbits. J. Pharmacol. Exp. Ther. 202:69-75, 1977.
- 13. Hackel, D.B., Ratliff, N.B. and Mikat, E.: The heart in shock. Cir. Res. 35:805-811, 1974.
- 14. Zar, J.H.: <u>Biostatistical Analysis</u>. Prentice-Hall, Inc., Englewood Cliffs, NJ, 1974.
- 15. Ettinger, S., Gould, L., Carmichael, J.A. and Tashjian, R.J.: Phentolamine: Use in digitalis-induced arrhythmias. Am. Heart J. 77:636-640, 1969.
- 16. Rothaus, K.O. and Powell, W.J.: The role of alpha adrenergic receptors in digitoxic tachyarrhythmias. Fed. Proc. 34:745, 1975.
- 17. Stickney, J.L.: The effect of different types of anesthesia on digitalis toxicity. Am. Heart J. 87:734-739, 1974.
- 18. Kelliher, G.J. and Roberts, J.: A study of the antiarrhythmic action of certain beta-blocking agents. Am. Heart J. 87:458-467, 1974.
- 19. Gillis, R.A., Pearle, D.L. and Levitt, B.: Digitalis: A neuroexcitatory drug. Circulation 52:739-742, 1975.
- 20. Weaver, L.C., Akera, T. and Brody, T.M.: Digoxin toxicity: Framery sites of drug action on the sympathetic nervous system.

  Tharmacol. Exp. Ther. 197:1-9, 1976.
- 21. McRitchie, R.J. and Vatner, S.F.: The role of arterial baroreceptors in mediating the cardiovascular response to a cardiac glycoside in conscious dogs. Circ. Res. 38:321-326, 1976.
- 22. Saxena, P.R. and Bhargava, K.P.: The importance of a central adrenergic mechanism in the cardiovascular responses to ouabain. Eur. J. Pharm. 31:332-346, 1975.
- 23. Dutta, S. and Marks, B.H.: Distribution of ouabain and digoxin in the rat and guinea pig. Life Sci. 5:915-920, 1966.

- 24. Roberts, J., Kelliher, G.J. and Lathers, C.M.: Role of adrenergic influences in digitalis-induced ventricular arrhythmia. Life Sci. 18:665-678, 1976.
- 25. Beeler, G.W. Jr.: Ionic currents in cardiac muscle: A framework for glycoside action. Fed. Proc. 36:2209-2213, 1977.
- 26. Brody, T.M. and Akera, T.: Relations among Na<sup>+</sup>, K<sup>+</sup>-ATPase activity, sodium pump activity, transmembrane sodium movement, and cardiac contractility. Fed. Proc. 36:2219-2224, 1977.

### Publications:

- 1. Korte, D.W. Jr. and Heiffer, M.H.: Apparent glycoside-specific antiarrhythmic activity of S-2-[5-(aminopentyl)amino ethyl] phosphorothioic acid (WR 2823). Pharmacologist 18:169, 1976.
- 2. Chung, H. and Brown, D.R.: The mechanism of the effect of acute stress on hexobarbital metabolism. Tox. Appl. Pharm. 37: 313-318, 1976.
- 3. Lowensohn, H.S., Khouri, E.M., Gregg, D.E., Pyle, R.L. and Patterson, R.E.: Phasic right coronary artery blood flow in conscious dogs with normal and elevated right ventricular pressures. Circ. Res. 39:760-766, 1976.
- 4. Korte, D. Jr., Heiffer, M. and Herman, E.: Characterization of the initial cardiovascular responses of S-2-[5-(aminopentyl)amino] ethyl phosphorothioic acid (WR 2823). Pharmacologist 19:243, 1977.

RESEARCH	AND TECHNOLOG	Y WORK UNIT	SUMMARY		OC 6445	77 1		Committee of the Party of the P	ONTROL SYMBOL &E(AR)636
3. DATE PREV SUMPRY		S. SUMMARY SCTY	6. WORK SECURITY	7. REGRA	DING DA	NS8'N INSTR'	ON TRACTO		. LEVEL OF SUM
76 10 01	D-Change	U	U	NA	1	NL	X YES	□ №0	A. WORK UNIT
IO. NO./CODES:*	PROGRAM ELEMENT		T NUMBER	TASK A	REA NUMBER			IT NUMBER	
L PRIMARY	61102A	3M161102E	3S01	0	00		123		
b. CONTRIBUTING									
c. CONTRIBUTING	CARDS 114F								
2. SCIENTIFIC AND TE	ical Research CHNOLOGICAL AREAS <sup>®</sup> hemistry 003			9	012900 I	Physiol	ogy		
			PLETION DATE					00	
76 07		CONT		DA		C. In-H			
a DATES/EFFECTIVE: NA EXPIRATION:			18. RESOURCES ESTIMATE		E & PROF	ESSIONAL MAN Y	HONAL MAN YRS b. FUNDS (In		
	NA	EXPIRATION:		1	77		7		394
C TYPE:		d AMOUNT:		FISCAL	CURRENT	-		-	334
& KIND OF AWARD:		f. CUM. AMT			78		6		326
. RESPONSIBLE DOD	RGANIZATION	1		20. PERF	ORMING ORGAN	ZATION			T -
NAME: Walter	Reed Army Ins	titute of	Research		Division	n of Bi	my Insti ochemist C. 2001	ry	Researc
NAME:	(202)576-3		COL, MC	HAME:*	DOCTOR,	B.P. )576-30	01	ic <u>InellAution)</u>	
TELEPHONE:	(202)370-3	,001		-	E INVESTIGATO		^		
	telligence No	+ 0		NAME:	WOLFE, A	ALAN D.	,		DA
	Telligence No	Tiongider		I MAME.	KILHUKI	SUIN FA	KI I		1)A

(U)cAMP (U)Antigen (U)Modulation (U)Polyamines (U)Acid Phosphatase

23.(U)The objectives of this work unit are to determine the rate and extent of cellular injury and regeneration as evidenced by measurement of macromolecules and their degradation products. The transport mechanism of molecules and their cellular interaction in tissue injury and regeneration will be elucidated. These studies are designed to define mechanism of healing combat wounds.

24.(U)The extent of cell death and the replication in the disease state will be measured either by macromolecules in biological fluids or their degradation products in urinary secretion. The biochemical diagnostic procedures which will aid in early detection of military significant diseases or the presence of causative agent will be developed. Several models are being investigated. (1) Prostatic acid phosphatase in bone marrow aspirates (2) Transport mechanism across the cell membrane and (3) Urinary secretion of macromolecular legradation products. The factors involved in directing the responses of lymphocytes in injury, infection, wound healing, and tissue rejection will be determined.

24.(U)76 10 - 77 09 The rate and extent of polyamine secretion under varied cellular degradation condition in a model system is measured and the results are evaluated.Radioimmunoassay for prostatic acid phosphatase is developed and is being used in clinical evaluation at WRAMC and several other clinics.Induction,isolation and partial purification of an enriched,antibody specific lymphoid cell fraction has been achieved.Uptake characteristics of drug by erythrocytes exposed to proteolytic enzyme have been carried but.For technical reports, see WRAIR Annual Progress Report 1 Jul 76 to 30 Sep 77.

235

Project 3M161102BS01 RESEARCH ON MILITARY DISEASE

Task Biomedical Sciences

Work Unit 123 Biochemical Research on Cellular Injury

Investigators.

Principal: Bhupendra P. Doctor Ph.D.

Associate: N.D. Brown, M.S.; Robert R. Copeland, B.S.; Larissa deBaare, M.D.; SP5 Piyush K. Gandhi,

B.S.; John A. Kintzios, B.S.; Sharon E. Koetitz B.S. P. Kuwabara; D.E. Mahan, Ph.D.; E. C. Richardson, M.S.; SP5 Duane C. Skar, B.S.; Mary A. Sodd, M.S.; A.D. Wolfe, Ph.D.

The objective of this work unit is the determination of the biochemical changes which occur during militarily significant trauma or cellular challenge and subsequent cellular and tissue regeneration. The following studies were conducted:

- 1. Determination of the changes in both macromolecular and specific metabolite fractions of lymphatic and other cells as a function of various cellular challenges.
- 2. Radioimmune assay development for analysis of prostatic acid phosphatase in bone marrow aspirates.
- 3. Investigation of theuptake characteristics of antimalarial drugs by erythrocytes in the absence of plasmodia.
- 1. Determination of the changes in macromolecular and specific metabolite fractions of lymphatic and other cells as a function of various cellular challenges.

Cell Interactions, Division, Growth and Death.

A knowledge of basic biochemical reaction involved in cell transformations would provide a better understanding of wound healing and tissue repair. One such particularly important system involves the related changes in the cellular concentrations of cyclic nucleotides and their related enzymes. These changes may be easily studied in lymphocytes responding to antigenic stimulas, i.e. the mixed lymphocytes response (MLR) and graft versus host (GVHR) reactions. In these systems the concentration of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) respond to specific challenge, as well as to specific hormones, and, in turn, partially control DNA, RNA, and protein synthesis.

# A. Cyclic Nucleotides:

In order to measure intralymphocytic cyclic nucleotide levels we developed or modified previously reported methods. Ion exchange column chromatography was selected as the method that best lent itself to rapid multiple sample determinations. Numerous different resins, Dowex 50X4, chelex 100, Bio-Rex 70 and polyethylenimedine (PEI), column dimensions, resin volumes and eluting combination were evaluated. Although all resins had the capacity to separate nucleotides to some degree, only one system was found to be compatible with our sample preparation. This system separated cAMP and cGMP on a Dowex 50X4 column and purified and concentrated each nucleotide on sequential columns of alumina. Currently methodology is being developed to adapt this method of analysis to microcultures of the MLC reaction. Also this methodology will be used to determine cAMP and cGMP levels in the spleens from graft versus host experiments.

### B. Polyamines

This project is a collaborative study with the Department of Urology, WRAMC, to determine if urinary polyamine levels accurately reflect the presence or absence of genitourinary cellular damage or allograft rejection. The polyamines, putrescine, spermine, spermidine, cadaverine and the diamines, 1, 3-diaminopropane and 1,2-diaminoethane will be measured in an amino acid autoanalyzer and the values obtained will be compared to the urine creatinine levels for a 24-hr. period. To date, over 391 urine samples have been collected, hydrolyzed in duplicate and creatinine levels determined with the final correlations to be made upon the completion of the polyamine analysis. Some preliminary results are described. A methodology was developed for the automated analysis of polyamines and diamines in urine. A highly specific analytical system with a reiterated tape program and dual columns offered several advantages over other methodologies. Ninhydrin-positive compounds, which are difficult to separate by other procedures, are quantitated by this method. Samples which contain 1,2 diaminoethane, 1,3 diamino propane, 1,4 diaminobutane (putrescine), 1,5 diaminopentane (cadaverine), spermidine, and spermine are separated in 90 minutes using an overlapping time sharing system. This approach to assaying diamines and polyamines in urine and serum specimens has been found to be applicable to characterizing various polyamines in the analyzed sample is indicative of the physiologic changes existing at that time, within the patient. Tentatively, we have observed levels of 1,2 diamino-ethane, 1,3 diamino propane, and cadaverine to be biological markers for cellular change during the growth of tumors and during the refection process of transplanted kidneys. The preparatory technique of the sample

is simple and the analysis of the sample relatively specific and sensitive. Further proof of our finding in verifying these early data will be of importance in better understanding the mechanics of the body in producing cellular changes by cancer the refection process.

### Conclusion:

We have developed a new methodology of extraction and analysis of urinary polyamines by an amino acid analyzer which allows operating pressures, reduced buffer flow and ease of operation. We have found for the first time two new compounds in the urine of patients with carcinoma of the genitourinary system. Both of these compounds in addition to spermine, spemidine and putrescine are elevated in some patients with genitourinary carcinoma. We believe that these compounds may be used as tumor markers in the diagnosis, staging and prognosis of patients with genitourinary carcinoma.

# 2. A radioimmunoassay for prostatic acid phosphatase.

Work during the last report year has centered upon evaluating the clinical usefulness of prostatic acid phosphatase (PAP) determination by radioimmune assay (RIA).

# Objectives:

- a. To establish the normal range of PAP in serum and bone marrow using RIA methodology.
- b. To establish the duration and magnitude of serum content of PAP following transurethral prostatictoring (TURP) and prostatic massage using RIA and enzymatic analysis.
- c. To asses the diagnostic usefulness of bone marrow PAP measurement by enzymatic and immunological procedures for confirmation of occult metastatic carcinoma of the prostate.

### Progress and Results:

#### Normal Range

(a) Serum PAP has been determined by RIA in 226 patients without prostatic disease. In this population serum PAP was found to be distributed in a nongaussian manner with a mean of 3.0 ng ml and a standard deviation of 2.4. An upper limit of 9.0 ng ml has been established for this group by non-parametric analysis.

(b) Serum PAP has also been determined in 186 patients with benign prostatic hyperplasia (BPH). As with the previous group, PAP was destributed in a non-gaussian manner with a mean of 2.9 ng/ml and a standard deviation of 2.3 in the BPH population. An upper limit of 9.7 ng/ml has been established for the BPH population by non-parametric analysis.

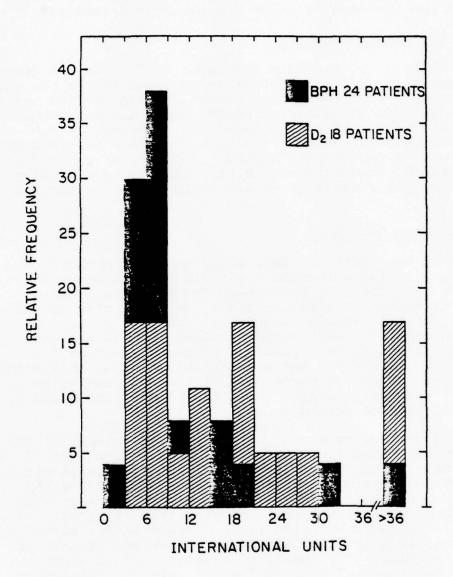
# II. TURP and Massage Studies.

In a study of 20 patients following TURP it became clear that all experienced a significant serum elevation of PAP following surgery. When analysis of serum PAP was carried out by RIA, the magnitude and duration of the elevation was proportional to the volume of tissue resected. However, little correlation was observed when the same serum was analyzed enzymatically. In contrast to the TURP studies, only one of 10 patients studied demonstrated a significant serum elevation either by enzymatic analysis or RIA following prostatic massage.

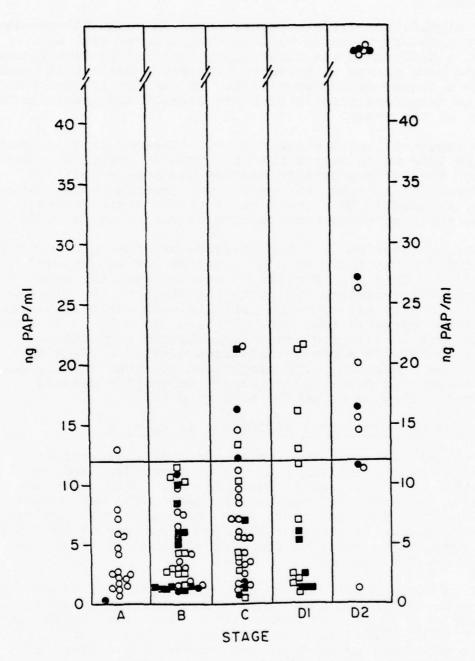
# III. Bone Marrow Studies.

Early in the course of this study bone marrow acid phosphatase was analyzed enzymatically using 1-naphthol phosphate as a substrate. However, because of the high incidence of false positive results (50%) obtained, we found this method to be of little diagnostic usefulness. The PAP content of bone marrow aspirates has been determined from 88 patients with benign prostatic hyperplasia. An upper limit of 13.7 ng/ml for bone marrow PAP has been established for this group and represents true 97.5 percentile of the BPH population. The mean of this distribution was 5.8ng/ml and a standard deviation of 3.6 was determined.

To date we have evaluated by RIA the PAP content of 183 bone marrow aspirates from patients with various stages of prostatic carcinoma. Ninety percent of those patients with proven bony metastatic (advanced) disease had elevated PAP values (greater than 13.7 ng/ml. Approximately 25% of this group had bone marrow PAP levels greater than 50 ng/ml. In contrast, in patients with localized disease (A+B), elevations were observed in only 4% of the cases. However, in patients with advanced clinically localized disease (C+D1), approximately 28% had elevated bone marrow PAP values. Clinically a significant proportion of the patients in this group (C+D1) can be expected to develop metastatic disease.



Shows the extent of overlap and blurring among different groups of patients when enzymatic assays for PAP are used as a diagnostic test.



Shows the distribution of bone marrow acid phosphatase levels determined by radioimmune assay from patients with benign prostatic hyperplasia (BPH) or carcinoma of the prostate by stage (A,B,C,D $_1$ , or D $_2$ ). Open data points ([ ]) indicates aspirates drawn from the sternum. Closed data points ([ ]) represent aspirates drawn from the iliac crest.

### Discussion:

No singificant difference was noted in serum PAP between patients without prostatic disease and patients with BPH. However, a difference was observed in the distribution between serum and bone marrow in patients with BPH. While this would indicate a degree of non-specificity in the RIA it does not appear to interfere greatly with the overall diagnostic performance of the assay.

As expected, serum elevation were observed in all cases following TURP using either RIA of enzymatic analysis. Serum elevation following prostatic massage has been a point of controversy for a number of years. Our experience indicates that it is possible to elevate serum PAP by rectal massage, Although the percentage experiencing elevation is low (10%).

Poor coorelation with the presence or absence of prostatic adenocarcinoma was found when bone marrow PAP was assayed enzymatically (Fig 1). Results of bone marrow PAP determinations by RIA correlate well with the stage of the disease (Fig II). Nearly all of those patients with advanced disease demonstrated elevated bone marrow PAP, while only 4% of those patients with localized disease demonstrated elevations. In those patients with advanced localized disease a 25% elevation frequency was observed. The elevations observed in this group may represent the detection of occult metastatic disease. Additional follow-up is needed to verify this.

# 3. Studies on Chloroquine Resistance in Malaria

Presently we are unable to prevent the emergence of resistance towards chloroquine (CQ) by plasmodia, and we know little of the molecular basis for it. Although plasmodia have not exhibited resistance towards the new antimalarial drug, mefloquine, there is no assurance that resistance might not eventually occur. Therefore a variety of considerations suggest the value of studying plasmodial chemotherapeutic drug resistance.

It is known that: (a) CQ-sensitive plasmodia cause the erythrocyte to take up more CQ than do resistant plasmodia (Macomber et., al., 1966, Fitch, 1970), (b) erythrocytes infected with CQ-resistant plasmodia lack a high affinity binding site possessed by erythrocytes infected by CA-sensitive plasmodia (Fitch, 1970), (c) the uptake of CQ by parasitized erythrocytes requires energy (Polet, 1969), (d) the amount of CQ taken up by a parasitized erythrocyte is function of both the plasmodium and the species origin of the erythrocyte

(Degowing and Powell, 1965, Fitch, 1970), and (e) competition and, therefore, structural specificity exists with respect to the uptake of CQ by psrasitized erythrocytes (Polet, 1969: Fitch, 1972). Consideration of these observation has led Fitch (1974) to studies on the uptake of CQ by uninfected erthrocytes exposed to the proteolytic enzyme, pronase, acting as a plasmodial substitute. Exposure of the erythrocytes to pronase caused a stimulation of CQ uptake, but such experiments failed to test the specificity of this process or its energy dependence. It was therefore decided to use this model system to begin studies of plasmodial resistance to CQ and a series of experimental were carried out to determine the specificity and other characteristic of this process of attachment. The experimental procedure involved incubation in 1% glucose- type B human blood with pronase for 15 minutes at  $37^{\circ}\text{C}$ , addition of  $(^{14}\text{C})$ -CQ in the absence or presence of a 200-fold excess of  $(^{12}C)$ -CQ, and continued incubation for a further 15 minutes. Erythrocytes were then washed free of excess drug by three consecutive centrifugations and resuspensions in aliquots of isotonic saline, and bound drug was extracted by incubation of erythrocytes with hot 5% trichloroacetic acid, or sodium hydroxide followed by a mixture of octanol and heptane. Radioactivity was assayed by liquid scintillation. The following results were obtained: (a) erthrocytes incubated with pronase bound twice as much CQ as did pronase-free erythrocytes, (b) non-radioactive carrier CQ reduced this uptake to the level of the pronase-free cells, (c) exposure of erythroctyes to ribonuclease after incubation with pronase appreciably reduced CQ binding, and (d) sulfhydryl group reagents yielded varied results. Table I presents the averages and standard deviations of experiments concerned with the specificity of CQ binding :

Table 1

	( <sup>14</sup> C)-CQ	BOUND (cpm)				
		+ Pronase	-Pronase			
$+(^{12}C)-CQ:$		1328 ± 520	1452 ± 971			
-( <sup>12</sup> C)-CQ:		2378 ± 1701	1277 ± 800			

These results suggest that the erythrocyte-pronase system might be improved to serve as a model system for a study of the uptake of CQ, but the high standard deviations indicate the poor reliability of present experiments.

Consideration of the above results and of the objectives of these experiments has led to a continuing attempt to develop the plasmodial growth system to Trager et al, (1975).

In this system, plasmodia undergo repeated subculturing and replication in vitro, thus providing an animal-free system with which to study plasmodial control of CQ uptake by erythrocytes. Several such week-long experiments have been conducted; 10 ml of diluted human type B blood were incubated at 37°C with a 10 µl inoculum of Aotus virgatus blood parasitized with Cq-resistant P. falciparum; the human blood sample had been diluted by 50% with Ringer's solution, selected nutrients, and (14 C)-deoxyadenosine prior to incubation and the aerobic atmosphere had been replaced with an atmosphere of 7%  $\rm CO_2$ , 5%  $\rm O_2$ , and 88%  $\rm N_2$ . A similar culture medium free of plasmodia served as a control. Each aliquot also contained 30 µg streptomycin per ml to prevent bacterial contamination. The growth of plasmodia was assayed daily through determination of the incorporation of (14C)-deoxyadenosine, and through the occurrence of additional parasitemia observed under a ligh microscope. Unfortunately little uptake of label occurred although some increase in ring forms was noted. This might become a valuable system with which to study plasmodial control of CQ uptake by erthrocytes when larger inocula become available, since the thousand-fold dilution of parasitized blood employed in these experiments renders difficult the occurrence and assay of growth and replication of plasmodia. Appreciable reduction in the volume of human blood inoculated will not permit isolation of transport proteins or of erythrocytic binding sites.

#### LITERATURE CITED

- 1. Macomber, P.B., O'Brien, R.L. and Hahn, F.E.: Chloroquine: Physiological basis of drug resistance in Plasmodium berghei. Science 152: 1374. 1966.
- 2. Fitch, C.D.: Chloroquine resistance in Malaria: A Deficiency in Chloroquine Binding. Proc. Nat. Acad. Sci. U.S. 64: 1181-1187. 1969.
- 3. Polet, H.: Uptake of chloroquine-3-H3 by plasmodium knowlesi in vitro. J. of Pharm. and Exp. Therap. 168: 187-192.
- 4. Fitch, C.: Plasmodium falciparum in Owl Monkeys: Drug Resistance and Chloroquine Binding Capacity. Science 169: 280-290. 1970
- 5. Degowin, R.L. and Powell, R.D.: Drug Resistance of a Strain of Plasmodium falciparum from Malaya. Amer. J. Trop.

- Med. and Hyg. 14: 519-527. 1965.
- 6. Fitch, C.: Chloroquine resistance in malaria: Drug binding and cross resistance patterns. Proc. Helm. Soc. Wash. 39: 265-271. 1972.
- 7. Fitch, C.: Chloroquine accumulation by erythrocytes: a latent capability. Life Sciences 14: 2441-2446. 1974.
- 8. Trager, W., and Jensen, J.B.: Human malaria parasites in continuous culture. Science 193: 673-675. 1976.

#### Publications:

- 1. Belville, W.D., D.E. Mahan, H.D. Cox, B.T. Mittemeyer, and A. S. Buck: Quantitative radioimmunoassay for human prostatic acid phsphayase. Abstract to American Urological Association, 72nd Annual Convention, 1977.
- 2. Bruce, A.W., A. Morales, W.D. Belville, D.E. Mahan: A comparative study of biochemical and immunological assays of acid phosphatase in carcinoma of the prostate: correlation with stage and grade. Abstract American Urological Association 72nd Annual Convention, 1977.
- 3. Bruce, A.W., A. Morales, W. Belville, D. Mahan: An objective look at acid phosphatase determinations: a comparison of biochemical and immunological methods. Abstract, British Urology Association, 1977.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY			DA OC 6444		2. DATE OF SUMMARY		REPORT CONTROL SYMBOL			
					77 10	01	DD-DR&E(AR)636			
3. DATE PREV SUM'RY	4. KIND OF SUMMARY	S. SUMMARY SCTY	S. WORK SECURITY	7. REGRADI	HO DA	DISB'N INSTR'N	SE SPECIFIC	DATA-	S. LEVEL OF SUM	
76 10 01	D. Change	U	U	NA		NL		□ MO	A WORK UNIT	
10. NO./CODES:*	NO./CODES:* PROGRAM ELEMENT PROJECT NUMBER		NUMBER	TASK ARE	EA HUMBER		WORK UNI	-	•	
& PRIMARY	61102A	3M161102B3	501	00		124				
b. CONTRIBUTING										
c. CONTRIBUTING	CARDS 114F									
11. TITLE (Procede with	Security Classification Code	,•								
(U) Biochem	ical Research	on Militar	y Diseases	3						
12. SCIENTIFIC AND TE	CHNOLOGICAL AREAS®									
002300 Bioc	hemistry 01	0100 Microl	oiology							
13. START DATE		14. ESTIMATED COM	PLETION DATE	15. FUNDIN	18 FUNDING AGENCY		16. PERFORM	16. PERFORMANCE METHOD		
76 07		CONT		DA			C. In-House			
					CES ESTIMA	TE & PROFE	& PROFESSIONAL MAN YES		b. FUNDS (In thousands)	
& DATES/EFFECTIVE:	NA	EXPIRATION:		"	ECEDING					
P. NOMBER:*				FISCAL	77		6		391	
C TYPE:		& AMOUNT:		YEAR EU	MAENY				- > /	
& KIND OF AWARD:		f. CUM. AMT.			78		8		336	
19 RESPONSIBLE DOD O					MING ORGAN					
ADDRESS:*	Reed Army Ins		Research	Di	vision		hemistry		Research	
Washing	ton, D.C. 20	012		PRINCIPAL	INVESTIGAT	R (Fumioh SEA)	I II U.S. Academic	Inelitution		
RESPONSIBLE INDIVIDUAL RAPMUND, GARRISON, COL, MC (202)576-3551			NAME.® DOCTOR, B.P., Ph.D.  TELEPHONE: (202)576-0031  ROCIAL SECURITY ACCOUNT NUMBER:							
TELEPHONE:			ASSOCIATE INVESTIGATORS							
21. GENERAL USE							MOO			
Foreign Intelligence Not Considered			HAME: LYON, J.A., CPT, MSC HAME: OLENICK, JOHN C. Ph.D. DA							

(U)Toxin (U)Immunoglobulin Synthesis (U)DNA (U)Antigenic Proteins (U)Vaccines

23. TECHNICAL OBJECTIVE. 24 APPROACH, 25. PROGRESS (Fundah Individual perspeptha Identified by number. Proceeds least of each with Security Classification Codes,)

23. (U)The objectives of this work unit are to(1) in vitro synthesize, isolate and characterize antigenic proteins from infectious agents(2) characterize and determine mode of

terize antigenic proteins from infectious agents(2)characterize and determine mode of action of bacterial toxins and other products(3)in vitro synthesize immunoglobulins and (4)employ DNA DNA hybridization for identification of unknown clinical bacterial isolates. These studies are designed to facilitate the production of vaccines against infectious diseases of military relevance.

24.(U)A cell-free parasite protein synthesis system using components only from parasites will be developed. These proteins will be evaluated for antigenic properties for use in developing vaccines against parasites. Shigella toxin will be purified, characterized, its mode of action determined and antibodies against it will be produced. A cell-free system for synthesis of specific antibodies against protein will be developed. The antibodies thus synthesized will be compared with in vivo synthesized antibody and eventually employed for passive immunity. DNA DNA hybridization will be employed to characterize newly isolated clinical bacterial isolates.

25.(U) 76 10 - 77 09 A highly sensitive radioimmunoassay for morphine was employed to determine the rate of clearance from serum of morphine administered during surgery. Protein synthesizing systems from T. rhodensiense have been isolated from both membrane-bound as well as cytoplasmic ribosomes in order to investigate the mechanism of antigenic variability. Shigella toxin was found to inhibit in vitro protein synthesis in pro and eucaryatic species. Antibody against this toxin has been produced. A quantitative, reproducible colorimetric assay for cytotoxicity against Hela Cells has been developed for the first time. For technical reports see WRATA Annual Progress Report 1 Jul 76-30 Sep 77.

DD. \*\*\* 1498

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DO FORMS 1498A, 1 NOV 66 AND 1498-1, 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE. Project 3ML61102BS01 RESEARCH ON MILITARY DISEASES

Task 01 Biomedical Sciences

Work Unit 124 Biochemical Research in Military Diseases

Investigators:

Principal: Bhupendra P. Doctor, Ph.D.

Associate: Michael Dodds, Clarence Emery, B.S.; SP5 Steven E.

Engelsen; George R. Fanning, M.S.; Mary K. Gentry, B.S.; Friedrich E. Hahn, Ph.D.; SP5 Ronald W. Hill, B.S.; Brian W. Jarvis \*, Ph.D.; CPT Jeffrey A. Lyon, MSC; John G. Olenick, Ph.D.; Earl C. Richardson, M.S.; SP5 Duane C. Skar, B.S.; Mary A. Sodd, M.S.; Frank E. Stokes; CPT Michael R. Thompson, MSC; SFC Glennon C. Vaughn; Diana Whitaker; Alan D. Wolfe, Ph.D.; SP4

Craig G. Zempel, B.S.

The objectives of this work unit are to determine macromolecular aspects of diseases of military importance in order to develop diagnostic and other procedures which will provide early detection, prophylaxis, or protection of military personnel from such diseases. The following studies were conducted:

- 1. DNA relatedness among Enterobacteriaceae.
- 2. Characterization of Shigella dysenteriae toxin.
- 3. Development of <u>in vitro</u> polypeptide synthesizing systems for determination and characterization of the mechanism of antigenic variability of Trypanosomes.
- 4. Drugs: Macromolecule Interaction
- 5. In vitro Biosynthesis of Antibody
- 6. Structure-Activity Relationships in the Chloramphemicol Series
- 1. DNA Relatedness Among Enterobacteriaceae

Enterobacteriaceae contain pathogens that cause a variety of disease including; enteric fever, diarrhea, urinary tract infection, food poisoning and bacteremia. In addition, <a href="Enterobacteriace">Enterobacteriace</a> contain phytopathogenic bacteria. The identification of pathogenic bacteria and the determination of their frequency of occurence, especially for atypical organisms and/or organisms not usually associated with disease, is an extremely important aspect of medical microbiology. Our studies are designed to determine DNA relatedness in all organisms in this family of bacteria. The data obtained are used for the following purposes:

respectively. Reassociation between DNA from the indigenous strain NZP 5105 and other indigenous strains averaged 64%. The proportion of non-complementary bases included in reassociated heterologous duplexes was determined from the melting profiles of the duplexes formed at 65° C. Reassociated duplexes from R. trifolii contained about 1% of noncomplementary base pairs. Reassociated duplexes of DNA form CC809, CC811, and NZP5105 with DNA from indigenous strains contained an average of 9.0, 8.5, and 5.6% of noncomplementary base pairs respectively. Base sequence homologies did not substantiate the distinction between primary clusters containing New Zealand indigenous rhizobia proposed by the phenetic classification, but they confirmed that indigenous rhizobia are more closely allied with acid producing R. lupini than with R. trifolii. The relationships among rhizobia are discussed in relation to those reported for the genus Agrobacterium in which the genetic composition of the population is much more clearly understood than it is for Rhizobium.

"This investigation was carried out in collaboration with Dr. B.D.W. Jarvis, Dept. of Microbiology and Genetics, Massey University, New Zealand during his visit to WRAIR.

#### 2. Characterization of Shigella Dysenteriae Toxin

#### (a) Biochemical Studies

From previous investigations on shiga toxin studies a highly enriched toxin preparation was obtained from S. dysenteriae 1 cell extracts. The active preparation is judged to be approximately 40% pure by analytical polyacrylamide gel electrophoresis. It demonstrated cytotoxicity to Hela cells (but not to Y-1 adrenal cells), neurotoxicity in mice, enterotoxicity in ligated rabbit ileal loops, and inhibition of in vitro mammalian and bacterial protein synthesis. Furthermore, analytical polyacrylamide gel electrophoresis of this material derived from a preparative isoelectric focused fraction separated cytotoxicity into two fractions, only one of which is mouse neutrotoxic. Ileal loop activity appears to correspond with cytotoxicity. The lability of these single band preparation has so far precluded determination of the fraction(s) responsible for in vitro inhibition of protein synthesis. Preliminary SDS electrophoresis of 125I-toxin and unlabeled toxin indicates the existence of subunits.

Material from non-denaturing analytical gel bands which demonstrated toxin activity were isolated and used for immunizing animals in order to obtain antisera. It was essential to couple the toxin with antisera prepared against crude cell extract in order to circumvent the high level of toxicity of toxin in rabbits. Rabbits received multiple injections of coupled toxin in complete Freunds adjuvant. The toxin elicited a response producing antibody capable of neutralizing cytotoxicity. Further characterization of toxin antibody is in progress.

#### (b) In Vivo Studies

of diversity between species of proteae can be taken as a basis for arguing that proteae be placed in a separate family. Such a decision is unjustified because proteae share the morphological and biochemical properties of the family, and because conserved DNA sequences, such as those that specify ribosomal ribonucleic acid, are highly related between proteae and other enterobacterium and substantially less related between enterobacteria and members of other families.

#### Characterization of Yersinia Ruckeri, the RM Bacterium

Cultures of RM (red mouth) bacterium, an etiological agent of red-mouth disease in rainbow trout (Salmo gairdneri), were characterized by means of their biochemical reactions, by DNA hybridization, and by determination of guanine and cytosine (GC) ratio in DNA.

The DNA relatedness studies have confirmed the fact that the RM bacteria are Enterobacteriaceae and that they comprise a single species which is not closely related to any other species of Enterobacteriaceae. They are about 30% related to species of both Serratia and Yersinia. Biochemical tests have shown the RM bacteria to be more closely related to yersiniae than serratiae.

The GC ratios of RM bacteria were approximately between 47.5% and 48.5%. This GC range is similar to that of yersiniae but markedly different from that of serratiae. On the basis of their biochemical reactions and their GC ratios the RM bacteria are classified as a new species of Yersinia, for which the name Yersinia ruckeri is proposed.

Phenetic Similarity and DNA Base Sequence Homology of Root Nodule Bacteria From New Zealand Native Legumes and Rhizobium Strains From Agricultural Plants

A comparison was made between 65 strains of root nodule bacteria from indigenous New Zealand legumes and 45 reference strains including: Rhizobium trifolii, R. phaseoli, R. leguminosarum, R. meliloti, and both "acid-producing" and "non-acid-producing" strains from the Lotus-Lupinus-Ornithopus cross-inoculation group. The strains were classified into 10 clusters on the basis of 37 morphological, cultural, and physiological tests. Relationships disclosed among the reference strains were in accordance with current ideas on Rhizobium taxonomy. The indigenous strains were well separated from both the trifolii-leguminosarum-phaseoli complex and R. meliloti but acid-producing strains from the Lotus-Lupinus-Ornithopus cross-inoculation group segragated with the indigenous strains. The principal characters differentiating the clusters are discussed. The base composition of DNA from representing indigenous strains was determined and base sequence homology studied. The relative reassociation at 65° C in 0.28M phosphate buffer between DNA from R. trifilii TAl and DNA from R. lupini strains and 9 indigenous strains CC809a or CC811 and indigenous strains averaged approximately 55% and 62% these strains are a new species and are tentatively classified as Escherichia ewingella. These results further demonstrate the biochemical variability in E. coli strains and illustrate the danger of identifying clinical isolates on the basis of a few biochemical tests.

#### DNA Relatedness Among Erwiniae And Other Enterobacteria

Relatedness of pectobacteria pathogenic to corn, grass and sugar cane, and of isolates of Pectobacterium chrysanthemi pathogenic for chrysanthema, dahlia, and dieffenbachia was determined by means of DNA reassociation. DNA relatedness data indicate that corn stalk rot bacteria and strains pathogenic on grass are highly interrelated. Furthermore, strains of both types are distinct from P. carotovorum and are most closely related to P. chrysanthemi. These strains are approximately 20% related to most enteric bacteria. P. chrysanthemi strains fall into two relatedness groups, one which contains isolates from chrysanthemum and quayule and the other, isolates from dahlia or dieffenbachia. A sugar can strain is 65% related to both the corn stalk rot bacteria and to strains of P. chrysanthemi. It is suggested that all of these organisms be considered as P. chrysanthemi and the corn stalk rot and grass isolates be designated P. chrysanthemi; pathovar zeae.

### DNA Relatedness In Species of Proteus And Providencia

DNA reassociation was used to determine relatedness among Proteus and Providencia and between these organisms and other enterobacteria The results indicate that:

- a. <u>P. mirabilis</u>, <u>P. morganii</u> and <u>P. stuartii</u> are homogeneous species.
- b. P. vulgaris, P. rettgeri and P. alcalifaciens each contain more than one relatedness group.
- c. A group of urea-positive strains previously called <u>P. rettgeri</u> biogroup 5 are in fact <u>P. stuartii</u>.
- d. Proteus myxofaciens is a valid species.
- e. Proteae are only distantly related to all other enterobacteria.

Taxonomic revisions consistent with these results include a 3 genus proposal with the genus Proteus containing P. mirabilis, P. vulgaris and P. myxofaciens; a separate genus for P. morganii; and the genus Providencia containing P. alcalifaciens, P. stuartii and Proteus rettgeri.

In an evolutionary sense the proteae are at the fringe of the family Enterobacteriaceae. Relatedness between proteae (except for P. morganii) and other enterobacteria is between 5% and 15%. This very low level of relatedness to other members of the family, coupled with the large degree

- (a) To identify atypical clinical isolates for purposes of epidemiology and treatment.
- (b) To develop a classification based on genotypic relatedness in place of phenotypic characteristics.
- (c) To develop a molecular definition of a bacterial species.
- (d) To accurately classify newly described bacteria.
- (e) To assess the lines of evolutionary divergence in pathogenic bacteria.

Previous data have indicated that enteric bacteria are all genetically related. The majority of cases show a "core relatedness" of about 20-25% among these organisms. The one general exception is found in the genera Proteus and Providencia. Most of these organisms exhibit 10-15% relatedness to members of all other genera.

During the past year we have concentrated on assessing relatedness within specific genera of medical importance, clarifying the status of recently described organisms, as well as atypical organisms, and on studies involving the evolutionary conservation of RNA cistrons. The majority of our research is in collaboration with Dr. Don J. Brenner, Center for Disease Control, Atlanta, Ga.

#### Conservation of t-RNA and 5S RNA Cistrons in Enterobacteriaceae

The genes for transfer ribonucleic acid (tDNA) and 5S ribonucleic acid (5S DNA) were isolated from the total DNA of E. coli. The relatedness of tDNA and 5S DNA from E. coli and other species of Enterobacteriaceae was determined by reassociation of the isolated genes labelled with  $^{32}\text{PO}_{\text{L}}$  to unlabelled, unfractionated DNA. Double-stranded DNA was separated from unreacted DNA by hydroxyapatite chromotography. Thermal elution profiles were done to determine the amount of unpaired bases present in related sequences. Both 5S DNA and tDNA, relative to total DNA, were highly conserved throughout the Enterobacteriaceae, including the genera Yersinia and Proteus.

## Genetic Similarity in Biochemically Atypical Clinical Isolates of E. coli

Previous DNA hybridization studies have shown that <u>E. coli</u> strains from diverse sources of isolation and geographical areas were at least 75% related. DNA hybridization and hydroxyapatite chromatography were used to determine DNA relatedness between atypical "<u>E. coli</u> like" clinical isolates and typical <u>E. coli</u>. Except for yellow-pigmented KCN+ strains, all of these organisms were 90% or more related to <u>E. coli</u>. The yellow-pigmented KCN+ strains were 87% to 90% interrelated and were 40% to 45% related to typical <u>E. coli</u>. As determined by DNA relatedness studies,

Consistent with the theory that Shiga toxin is a natural constituent of the bacterium, experiments were undertaken to determine the time course of toxin synthesis relative to bacterial growth; synthesis of toxin was found to occur in direct proportion to growth, and, moreover, maximum recovery of toxin occurred upon alkaline extraction of the bacteria rather than upon isolation from the bacterial culture medium. Thus the toxin appeared to be present in direct ratio to other cellular components, and not readily released by the cell. Both observations reinforce the idea that the toxin is a natural constituent of these bacteria.

## (c) Universal Existence of Shiga Toxin in Enteropathogens

One possibility regarding the role of toxin in pathogenicity is that almost all, if not all enteropathogens causing diarrhea produce shiga like toxin. This hypotheses has been tested at the present. The procedures developed for purification of Shiga toxin has been employed to enrich the toxin from many non-pathogenic as well as pathogenic strains of E. Coli. The toxin from E. Coli is "shiga like toxin", as evidenced by (1) it is neutralized by antisera against shiga toxin (2) physicochemical parameters related to column chromatography, gel filtration and some electrophoretic behavior, (3) cytotoxicity in Hela cells and, (4) enterotoxicity in rabbits. Systematic studies in various phases of this investigation is carried out in the Dept. of Bacterial Diseases in collaboration with the Div. of Biochemistry.

### (d) A Novel Cytotoxicity Assay

Cytotoxicity to Hela Cells has commonly been employed as an index of activity of bacterial toxins and viruses. The present acceptable assay required confluent monolayers of cells in a tube containing a large number of cells. The detachment of cells is taken as an endpoint of the assay. The cost is very high and also the assay results depend on batch of cells, the conditions of experimentation and other parameters. This makes the assay irreproducible from day to day, person to person, and from laboratory to laboratory. The modification of this assay, as worked out, not only eliminates most of these variables but also makes possible for the first time quantitation of activity. The modified assay procedure is briefly described here. The assay utilized microtiter plates and cell concentrations of 2-3 X 10<sup>4</sup> cells/0.1 ml/well. Toxin dilutions and cell suspensions in appropriate medium are incubated together in microtiter plates. At the end of the incubation period, remaining live cells are still attached to the bottom of the well, whereas dead ones are suspended in the medium. The dead cell suspension is aspirated off and the attached cells are stained with crystal violet. After rinsing off excess dye, the stained cells are extracted with alcohol and the absorbance of the extract is measured spectrophotometrically to quantify the amount of live cells remaining in a given well.

A typical profile is shown in Figure 1. These profiles can be analyzed in several ways.

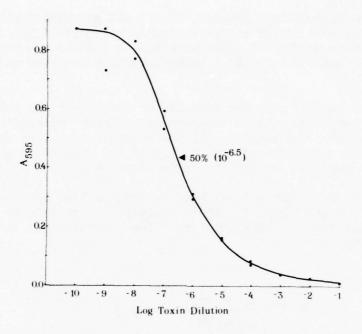


Figure 1: Semi-log plot of dye uptake vs. toxin dilution

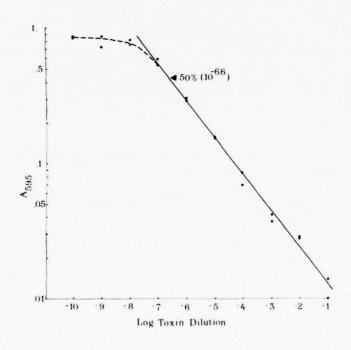


Figure 2:Log-log plot of dye uptake vs. toxin dilution

- (I) If negative control samples, (i.e., wells containing cells with no toxin) are included in the assay, a graphic estimate of the toxin dilution correspondence to the 50% absorbance can be made from a semi-log plot, the figure of 50% cell death (LD $_{50}$ ) being interpreted as the end-point of the assay.
- (II) From a log-log plot the most linear portion of the curve can be chosen (solid line, Figure 2); a least-squares linear regression of the form y=a+bx will give the equation for the best straight line and the point on that line which corresponds to 50% of the negative control value indicates the end-point of the toxin dilution.
- (III) Alternately, all points from the complex log-log plot (solid line plus dashed line, Figure 2) can be analyzed iteratively by computer to give an estimate of the  $LD_{50}$  dilution. The general model for this solution is of the form,

$$y = \frac{a+d}{1+\left(\frac{x}{C}\right)^b} + d$$

where a is the absorbance at minimum cell death, d is the absorbance for maximum cell death, b is the "steepness" of the curve, and c is the toxin dilution corresponding to the  $LD_{50}$  (a+d/2). The advantages of this method are two-fold: it is not necessary to include negative controls on the microtiter plates and the method used to analyze the curves allows confidence limits to be placed on the estimates of the parameters. Nonetheless, agreement between the methods is good, the toxin dilution resulting in  $LD_{50}$  for the assay shown being estimated at  $10^{-6.5}$  (graphic method),  $10^{-6.63}$  (linear regression technique), and  $10^{-6.52}$  (nonlinear least squares regression).

This assay therefore provides a new and sensitive procedure for quantitation of the potency of toxins, drugs, and viruses.

# (e) Action of Shiga Toxin on In Vitro Polypeptide Synthesizing Systems of Prokaryotic Origin

Previous studies showed that Shiga toxin inhibits polyphenylalanine synthesis in cell-free systems derived from eukaryotes. In view of the possibility, however, that the toxin may be normally a functional constituent of Shigella dysenteriae as well as of other enteric bacteria, it became of great interest to extend our investigation to include determination of the influence of Shiga toxin on similar polypeptide synthesizing systems derived from Escherichia-coli and Shigella dysenteriae. Increasing amounts of the toxin, preincubated with ribosomes prepared from either bacterial species, followed by addition of the conventional reaction components, produced almost identical degrees of inhibition of poly

U-directed synthesis of polyphenylaline. The property of the toxin to inhibit a cell-free system from <u>E. coli</u> was not unexpected, but that this inhibitory property extends to a system derived from the bacterium from which the toxin was obtained is unexpected, although not without precedent, since the <u>E. coli</u> ribosomal protein S 1 when purified and added exogenously to similar amino acid polymerizing systems is inhibitory (Jay and Kaempfer, 1974). Natural products out of situ may become inhibitors in systems in which they function by virtue of configuration and locus.

Heating the toxin for 5 minutes at 90° C almost totally destroyed its inhibitory action. Preincubation of other components of the reaction mixture, i.e., poly U, the enzyme supernatant fraction, or transfer RNA, with the toxin revealed only minimal degrees of inhibition when compared with inhibition achieved through preincubation of the toxin with ribosomes. However, increasing the time of preincubation of ribosomes with toxin did not increase the extent of inhibition: the ribosomes had merely to be preexposed to toxin. The results suggest that the toxin may react with, or bind to, ribosomes and that the ribosome may be the primary site of toxin action.

Experiments were performed to determine the effect of toxin on the kinetics or rate of formation of polyphenylalanine. Reaction mixtures were incubated at 30° C to slow the rate of synthesis and to prolong the linear phase of incorporation of radioactive-labelled phenylalanine into polypeptide. Toxin was added to an amount to produce approximately 70% inhibition. When pre-exposed to ribosomes, toxin decreased the rate of polyphenylalanine formation by a constant percentage. However, if the same amount of toxin was added to an assay mixture 5 minutes after the start of phenylalanine polymerization, the rate of synthesis remained unaffected. It appears that ribosomes programmed and synthesizing polyphenylalanine are not susceptible to toxin action indicating a preferential inhibition of initiation of protein synthesis.

Reaction mixtures, in which toxin was absent, or in which ribosomes had been pre-exposed to toxin, were analyzed both by light scattering and by sucrose density gradient centrifugation. Light scattering analysis of such reaction mixtures revealed that those systems containing toxin exhibited higher scatter than did the uninhibited systems. Comparison of sucrose gradients from toxin containing and toxin-free systems revealed that monosomes from the toxin containing reaction mixtures sedimented slightly more rapidly or possessed a slightly greater absorbance at 260 nm than did monosomes from toxin-free mixtures. The light scattering characteristics and the sucrose density gradient characteristics of the respective systems are in agreement and suggest that the toxin prevents the ribosome from dissociating into its subunits as uninhibited ribosomes do. Sucrose gradients also showed that the toxin did not selectively inhibit polyphenylalanine synthesis by monosomes or polysomes; uniformly lower amounts of polyphenylalanine were observed throughout the gradients of the toxin-containing systems in comparison with the toxin-free systems.

# (f) Relationships of Shigella flexneri 2a toxin to S. dysenteriae 1 toxin.

A toxin extracted from heat inactivated, alkaline treated S. flexneri 2a showed biological properties similar to S. dysenteriae I toxin. The S. flexneri 2a toxin was lethal to mice, enterotoxic for ileal loops of rabbits and cytotoxic for Hela cells. A serological relationship between S. flexneri 2a and S. dysenteriae I toxin was shown with cross neutralization tests. The details of this investigation appears elsewhere in this annual report.

# (g) Effect of Shiga toxin on PHA Stimulated Lymphocyte Transformation

One of the proposed mechanisms of action of Shigella toxin was the inhibition of protein synthesis. In order to test this hypothesis lymphocyte cultures were prepared utilizing human peripheral blood lymphocytes stimulated with PHA mitogen. Concentrations ranging from  $10^{12}$  to  $10^3$  dilutions of a partially purified toxin of Shigella dysenteriae were added at the time of PHA stimulation (0 day) and at 4 days of culture. This allowed the testing of the toxin effects upon the transformation process and upon the actively synthesizing cell. A single preparation of toxin was used throughout the experiments. This lot was found to be toxic to Hela cells at  $10^{11}$  dilution. All cultures were pulsed for 18 hours with H-thymidine (for DNA determination) and  $^{11}$ C amino acid mixture (for protein determination) at 4 days of culture.

Of the five individuals tested, two did not have known prior exposure to S. dysenteriae or its toxins. One of the three remaining had an active case of shigella dysentery within the last three years, another had been exposed to the disease early in life because the disease was endemic to the area in which he had lived; the last individual had a current laboratory exposure from extracting and purifying shigella toxin. The toxin was found not to effect DNA or protein synthesis in the four day cultures of lymphocytes from unexposed individuals but was inhibitory for both protein and DNA synthesis at dilutions greater than 10 in zero day cultures. This suggests that the toxin may interface with the mitogen-induced transformation process in unsensitized individuals. In the three individuals with exposure histories the results are not so definite. Differences in response ranged from a stimulation of protein synthesis in zero and four day cultures to no effect. These observations are preliminary and thus no conclusion can be drawn from them.

Shiga toxin studies are carried out in collaboration with investigators in the Dept. of Bacterial Diseases and Virus Diseases, DCD&I.

3. Development of In Vitro Polypeptide Synthesizing Systems for Determination and Characterization of the Mechanism of Antigenic Variability of Trypanosomes.

Since it is possible that U.S. military personnel may be required to operate in areas where trypanosomiasis is endemic, a greater understanding of this parasitic disease is required in order to develop more efficient means of control, prevention, and clinical treatment.

An immunotherapeutic approach has been difficult and is hindered by the capability of the salivarian trypanosomes to evade the immune mechanisms of the infected host. In response to the successive production of specific antibodies, antigenically variable trypanosomes emerge sequentially in the blood causing waves of parasitemia in which the parasites arising in the blood of each succeeding wave differ antigenically from those of preceding waves.

It was first proposed by Vickerman (1969) that the surface coat of salivarian trypanosomes may be intrinsically involved in the phenomenon of antigenic variation. It is now known from the work of Cross (1975) that the variant antigen is a glygoprotein and is a major component of the trypanosome surface coat. Glyprotein surface antigens prepared from related, but antigenically different, trypanosomes have been shown (Bridgen, Cross & Bridgen, 1976) to be immune specific and to have completely different amino acid sequences. It appears, therefore, that the antigenic glycoproteins of variant trypanosomes are unique and are not simply minor modifications of a basic type.

It was also suggested by Vickerman (1969) that the extracellular surface coat is a secreted product of trypanosomal origin and not simply an accretion of host serum proteins. Taylor and Cross (1977) have demonstrated the cycloheximide and puromycin-sensitive incorporation of ( $^{35}$ S) L-methionine into the surface glycoprotein of trypanosomes maintained in a chemically defined medium indicating that the variant antigen is indeed biosynthesized and incorporated into the surface coat.

Although biochemical investigations have apparently provided some information on antigens and antigenic variation in the African trypanosomes, further studies are obviously required before the actual mechanism(s) involved in antigenic changes can be elucidated. Such knowledge would undoubtedly be of immense practical importance in the ultimate development and successful use of vaccines against African sleeping sickness.

The present studies were initiated to investigate the possibility of synthesizing the variant antigen in vitro by a cell-free ribosome-enzyme system isolated from a cloned strain CP3B3 of Trypanosoma rhodesiense. In the cytoplasm, ribosome either occur freely or are associated with the endoplasmic reticulum. It is generally accepted that the free cytoplasmic robosomes are employed in the biosynthesis of protein intended for internal use, whereas the ribosomes that are attached to the endoplasmic reticulum are responsible for making of proteins to be secreted outside the cell. Since available evidence suggests that the surface coat variant antigens are secreted or exported to the outside of the cell, it was decided to prepare both free and membrane-bound polyribosomes to

utilize these ribosomal preparations in a homologous cell-free protein synthesizing system, and to identify the synthesized proteins by immuno-coprecipitation with isolated and purified surface glycoprotein and specific antiserum.

Male rats (250-400 g, Walter Reed strain) were infected intraperitoneally with stabilates from a cloned strain CP3B3 of Trypanosoma rhodesiense. A parasitemia was generally achieved after 72-96 hours. Rats were anesthesized with ether and exsanguinated via the aorta using heparin as anticoagulant. The collected parasitized blood was centrifuged in 15 ml tubes for 10 minutes at 1500 g. Serum lying above the buffy layer of trypanosomes was removed and the parasites were carefully (to ensure minimal erythrocyte contamination) drawn into a pipette and applied to a column of DEAE-cellulose. Trypanosomes were eluted under conditions where host blood cells were retained. The purified trypanosomes were sedimented and further washed as required. Suspensions of trypanosomes, separated from parasitized rat whole blood by centrifugation and purified free of contaminating blood by passage through a column of DEAE-cellulose, were disrupted by homogenization with glass beads in a Sorvall Omni-Mixer. Free polyribosomes and membrane-bound polyribosomes were prepared from the broken cell suspensions using a discontinuous density gradient centrifugation technique. Conventional requirements for in vitro protein synthesis were employed in assaying the two polyribosomal preparations and radioactive labelled amino acids were indeed incorporated into proteins (trichloracetic acid-insoluble material), albeit at low specific activities. At the present time, the cell-free extracts, preparations of ribosomes and supernatant enzyme fractions, are being characterized and optimal requirements for the in vitro synthesis of proteins are being systematically determined in order to increase substantially the activities of the homologous systems. Biochemical studies such as these require a plentiful supply of trypanosomes. Therefore, efforts are being made to secure adequate amounts both through the isolation of trypanosomes from parasitized animals and by the in vitro culturing of animal-infective trypanosomes as described by Hirumi, Doyle & Hirumi (1977).

### 4. Drugs: Macromolecules Interaction

Studies on the inhibition of DNA synthesis by intercalating drugs, such as the antimalarials chloroquine and quinacrine, have been conducted in vitro (O'Brien et al, 1966), utilizing purified Escherichia coli polymerase I. Recently E. coli polymerase III has been shown to be essential for DNA replication in vivo. A demonstration that pol III would be inhibited by intercalants thus was necessary to support current concepts that these compounds prevent cell and plasmodial replication through suppression of DNA synthesis. DNA polymerase III from E. coli N211 pol A trypgal was purified by the method of Wickner and Kornberg (1974) and its activity was tested in the presence and absence of a selected group of antiplas-

modial and intercalating drugs and compounds. Enzymatic activity was suppressed by all the tested intercalants, and their activity varied directly with their planar area. Probit transformation analysis showed the inhibitions to be linear, suggesting a single mode of action. Preincubation of the enzyme itself with a concentration of ethidium bromide which vielded 50% inhibition of polymerization (7 X 10<sup>-5</sup>M) failed to enhance inhibition. The results led to three conclusions: (1) intercalants and intercalating antimalarial drugs inhibit polymerase II catalyzed replication of DNA, (2) one mode of action is involved, and (3) the mode of action involves drug DNA template or primer interaction.

#### 5. In Vitro Biosynthesis of Antibody

The project involves two approaches, (1) the humoral aspects of antibody production, and (2) the cellular aspect of antibody production.

(a) Humoral Aspects: A method has been developed which permits the conjugate of succinylmorphine and human serum albumin to be covalently bound to sheep erythrocytes. The binding was accomplished by a carbodii-mide reaction and caused only insignificant lysis of the erythrocytes. The derivated erythrocytes were sensitive to agglutination and complement fixation.

Several methods were established for obtaining total gamma globulin or anti-morphine gamma globulin at purities greater than 95% as determined by sodium dodecyl sulfate (DSD) electrophoresis: (1) Ammonium sulfate precipitation, as described by Campbell et al, (1974), followed by Agarose 5-m and Sephadex G-100 chromatography. (2) Specific absorption of Fc fragments to Staph A Sepharose and elution with 0.1M glycine HCl permits elution of pure gamma globulin. (3) A twenty fold or better purification of anti-morphine gamma globulin from total gamma globulin was achieved using succinylmorphine as an affinity chromatography matrix. Elution of the specific gamma globulin from the column was effected with 8 M guanidine HCl. (4) Preabsorption of serum albumin on Blue dextran Sepharose followed by chromatography on A-5M agarose was found to be unsatisfactory for pure gamma globulin isolation.

Studies are also being conducted on the protein biochemistry and the immunological activity of the isolated globulins. The loss of antibody activity on dialysis has been a continuing problem. The antibody activity losses are minimal when high concentrations of antibody are used. Morphine immune gamma globulin, after reduction with dithiothreotol and alkylated with iodoacetic acid, shows only light and heavy chains on SDS electrophoresis. The modified gamma globulin exhibits no loss of binding activity. The separation of the light and heavy chains by gel filtration in guanidine HCl is planned since methods for determining binding activity in the presence of guanidine HCl have been developed.

The filter technique for antigen-antibody reactions was further evaluated. To demonstrate that the filter binding of gamma globulins was not merely an artifact of rabbit anti-morphine gamma globulin, bind-

ing studies with <sup>3</sup>H dinitrophenyl (DNP) lysine, anti DNP (Sheep) and anti DNP (rabbit) were performed. The test complexes were bound by the filter. The method further demonstrated its utility, using age response experiments in mice. The selective binding of <sup>3</sup>H-morphine to immunized mouse globulin was observed. Experiments are being conducted to determine which portion of the anti-morphine gamma globulin is bound by the filter.

The filter binding method has been adapted to the assay of antimorphine gamma globulin in the presence of a large excess of competing non-labelled ligand. The protein plus non-radioactive ligand is applied to the filter, the filter is washed, incubated with an excess of labelled ligand, washed with a high ionic strength buffer, and counted for radioactivity. This method is also used for the detection of anti-morphine gamma globulin in the presence of denaturants.

(b) Cellular Aspects: There has been a developing interest in the study of the origin of antibody diversity, specialization of lymphocyte populations and basic mechanisms of immunoglobulin biosynthesis. Therefore, methods have been developed and focused on isolating cell-free immunoglobulin synthesizing systems. Most of the investigations have utilized the immune globulin producing murine plasmacytoma cells which serve as a source of the instructional molecule for immune globulin synthesis. The mRNA is able to induce in cell free extracts, derived from Ehrlich ascites tumor cells or wheat germ products that are antigenically and structurally related to the immunoglobulin produced by the intact myeloma. One of the limitations of the murine plasmacytoma immunoglobulin is a lack of known antibody function. It does possess an antibody-like affinity for dinitrophenyl and trinitrophenyl groups, but its binding constant for these groups is several orders of magnitude less than that associated with antigen-antibody reactions, indicating a probable non-antibody function.

The immuno-responsive cells, the lymphocyte and plasma cell are responsible for the biosynthesis of immunoglobulins. A population of lymphocytes, bone marrow derived, (B cells), contain receptor immune globulins in their outer membrane. Specific antigens attach to this membrane bound antibody or receptor site on the B cell and stimulate it to differentiate and proliferate into immunoglobulin producing and secreting plasma cells. Since the formation of antibody by a lymphocyte that is specific for an antigen or hapten indicates that it carries the instructional mRNA, it should be possible to isolate this molecule and produce specific antibody in vitro with a cell free protein synthesizing system. This project is being conducted in three phases:

- Phase I Production, isolation and purification of specific antibody lymphoid cells.
- Phase II Extraction, purification and translation of antibody specific mRNA.

#### Phase III <u>In-vitro</u> and <u>in-vivo</u> assay of cell free product.

The initial experiments in Phase I consisted of demonstrating a cellular immune response to the hapten, morphine. This hapten was selected because a humoral immune response had been demonstrated to it when it was succinylated and conjugated to bovine serum albumin, and a sensitive radio-immuno assay for it had been developed, thus facilitating the detection of the hapten or its antibody. Antigen stimulated cultures of spleen cells from C57 B1/6J mice that had been injected with saline, morphine sulphate (MS), succinylmorphine conjugated to bovine serum albumin (SM-BSA), or bovine serum albumin (BSA) in 50% complete Freund's adjuvant. The in-vitro stimulating antigens consisted of various concentrations of MS, SM-BSA and BSA and the mitogens phytohemagglutinin (PHA-P) and lipopolysaccharide (LPS). Labelled thymidine incorporation was employed as an indicator for cell responsiveness. It was found in the mitogen-stimulated cultures that there was a relatively greater response to PHA-P and LPS in cells from MS & SM-BSA injected mice than cells from saline and BSA injected mice with maximum values obtained at 6-7 days after injection. In-vitro lymphocyte reaction was also greater in MS and SM-BSA spleen cells compared to saline and BSA cells. These results indicate that MS when injected into mice is able to elicit a population of lymphocytes capable of being transformed by the stimulation of MS and this population of responsive cells exhibit a peak at 6-7 days.

#### 6. Structure-activity relationships in the chloramphenical series.

The antibiotic, chloramphenicol, is a drug of military importance. Its effectiveness against rickettsial infections (scrub typhus) was first demonstrated in Kuala Lumpur by a team of investigators from WRAIR. It remains the drug of choice in the treatment of typhoid fever and of otherwise rapidly fatal cases of bacterial meningitis. Its medical usefulness is compromised by its ability to cause aplastic anemia through an unknown mechanism in one case per 20,000 to 30,000 administrations. Plasmid-mediated resistance to chloramphenicol is a frequent occurrence, caused by acetylation of the two hydroxyl groups of the drug by plasmid gene products. The development of superior, plasmid-insensitive and non-toxic derivatives of chloramphenicol appears to be feasible.

For this purpose, the extensive body of structure-activity data on more than 500 synthetic derivatives and analogs has been collated and analyzed with a view to arriving at a prototypical structure which can guide successful molecular modification.

For the purpose of analysis, the molecule of the drug can be subdivided into three moieties I. The electronegatively substituted aromatic ring system, II. The acyl side chain and III. The 1,3-propane diol-2-amido side chain.

### CHLORAMPHENICOL

I. The aromatic ring system permits considerable variations with respect to the ring structure itself and to its substituents, resulting in compounds whose antibacterial activities are modified rather than abolished. The p-nitrophenyl group can be replaced by biophenyl, 4' - bromobiphenyl or 4' - methylbiphenyl without significant loss (and for Sarcina lutea even a gain up to 2.8 times) in antibacterial potency. The p-nitro-azobenzene derivative of chloramphenical approaches the antibacterial potency of the antibiotic itself. The p-SCH3 derivative, also has high antibacterial activity. Replacing p-nitrophenyl by 5-nitro-2-thienyl, 5-nitro-2-thienyl, 5-nitro-1-naphthyl or 4-pyridyl results in compounds with significant antibacterial activities. Evidently, the steric properties of the aromatic system are not of critical importance for the antibacterial action of chloramphenicals, except that ortho or meta substitution appears substantially to reduce potency.

The antimicrobial potency is a function of the relative electronegativity of the para substituent of the aromatic ring system as shown in Figure 2.

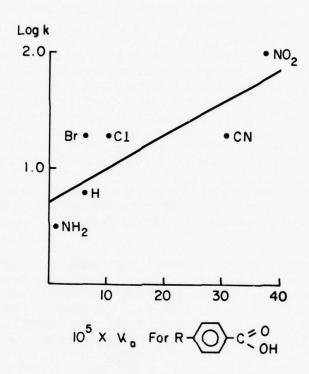


Figure II. The logarithms of the per cents reduction in bacterial (E.coli) growth rates by a series of chloramphenical derivatives plotted as a function of the electrolytic dissociation constants of correspondingly parasubstituted benzoic acids.

While the electronic properties of the substituents might be determinants of the antibacterial potencies of these derivatives, one should not exclude additional contributions from hydrophobic effects which may play a role in the penetration of the compounds into the bacterial cells and especially in the attachment to their site of action. Substitution in the para position of six ionogenic and, hence, hydrophilic groups abolished antibacterial activity.

II. The acyl side chain is essential for antibacterial potency; the deacylated chloramphenical base is almost devoid of activity. Variations of the acyl substituent have been the most frequently introduced alterations of the chloramphenical molecule. On the basis of insufficient growth inhibition data, Hahn had speculated that the electronegativities of the acyl substituent were determinants of antibacterial potency. When

this idea was reexamined, using inhibition data of Hansch, no satisfactory correlation was obtained. For example, chloramphenicol itself had a log k of 2.00 with dichloroacetic acid having a  $\rm K_{\rm Q}$  of 3.32 X  $\rm 10^{-2}$  and pk of 1.48, while the trichloroacetyl derivative had a log k of 0.75 with trichloroacetic acid having a  $\rm K_{\rm Q}$  of 2 X  $\rm 10^{-1}$  and a pk of 0.70. It should be noted that the electronic Hammett constants of for 20 chloramphenicol derivatives with different acyl residues, R, showed marginally significant correlation with the antibacterial potencies of these compounds. Moreover, the charges of 0,C,N and H for the acylamino sidechains reveal no detectable relationship to the antibacterial activities of these compounds. Not only the polarity but the polarizing action of the acyl substituent is important for antibacterial activity: the acyl substituent affects the acidity of the amide nitrogen proton. In fact, substitution of the amide H with methyl abolishes antibacterial activity.

Many workers agree that the steric properties of the acyl moiety limit the antibacterial action of chloramphenical derivatives and that increases in the size of this substituent decrease potency. Bulky substituents are undesirable.

III. The propanediol moiety possesses two asymmetric carbon atoms which give rise to four stereoisomers. Two of these, the D-threo (chloramphenicol) and the L-threo enantiomers, carry the amide side chain and the hydroxyl on carbon 1 on opposite sides of the plane of the two asymmetric centers, while the other two enantiomers, the L-erythro and D-erythro stereoisomers, carry the two substituents on the same side of this plane. Only chloramphenicol itself, the D-threo isomer, has strong anti-bacterial properties. Methyl substitution for the H on carbon atom 2 abolishes antibacterial activity. The absolute stereospecificity of chloramphenicol's action also applies to active derivatives of the antibiotic.

Substitutions of the two hydroxyls, exchanging them for H atoms or suppression or extension of the terminal -  $\text{CH}_2\text{OH}$  result in non-active compounds. Diacetylation of the hydroxyls is the phenotypic expression of R-factor determined resistance to chloramphenicol.

X-ray diffraction analysis of the dibromoacetyl derivative of chloramphenicol had shown that the two hydroxyls approach each other closely in the crystalline compound and suggested the formation of a hydrogen bond which closed a five-membered ring structure. Nuclear magnetic resonance spectroscopy seemed initially to support the same conclusion for chloramphenicol in partially aqueous solutions. However, when the conformation of chloramphenicol was reinvestigated by NMR and infrared spectroscopy, no evidence of hydrogen bonding in solution was obtained.

The earlier view of chloramphenical as forming a hydrogen-bonded fivemembered ring structure in solution led to speculations that the drug was alternately an analog of uridylic acid or of an amino-acylated nucleoside resembling puromycin. Both speculations had in common that they regarded the assumed hydrogen-bonded five-membered ring structure in chloramphenical to be analogous to ribose.

The structural variations which are permissible for the aryl moiety of active chloramphenical derivatives collectively weaken analogies of this aryl to uracil or dimethyl adenine. Observations on growth-inhibitory activities of several chloramphenical derivatives with covalently closed six-membered alicyclic systems were ambiguous. The analogy of the putative ring system to ribose has, however, been tested directly through the synthesis of the ribosyl derivative of chloramphenical, Fig. 3 (Klein, Lotick, Watanabe and Fox, 1971). This compound was totally non-active against 10 different bacterial strains and showed only insignificant effects on the growth of  $\underline{E}$ .  $\underline{coli}$  on plates.

Figure III

Fig. 3 Conjectural hydrogen-bonded ring structure in the chloramphenical molecule at left and structure of a ribosyl derivative of chlorambonice at right (Klein et al. 1971).

Intromolecular hydrogen bonding as a determinant of antimicrobial activity had already become doubtful after it was shown that replacement of the hydroxyl of carbon atom 1 by Cl or conversion of the 1-hydroxy into the 1-keto compound produced substances with appreciable antimicrobial activity. A tabulation of 40 derivatives having the 1-keto group (Kolosov et al., 1961) listed several actively antibacterial compounds. Most important, Fig. 4 shows two chloramphenicol 1-keto derivatives which are active in inhibiting the growth of Staphylococcus aureus (Kono et al., 1969). Intramolecular hydrogen bonding is excluded for both derivatives.

Figure IV.

#### SUPERIOR CHLORAMPHENICOL DERIVATIVES

$$\begin{array}{c|c}
O = C - CHCI_{2} \\
NH \\
O = C - C = CH_{2} - 60X \\
O = C - CHCI_{2} \\
NH \\
O_{2} N \bigcirc -C - CH - CH_{2}Br - 38X \\
O
\end{array}$$

It has been mentioned above that the plasmid-mediated inactivation of chloramphenical involves acetylation of both hydroxyl groups of the propane moiety. (See Figure 1). Two compounds (Fig. 4) were specifically developed to remove the target sites for acetylation by plasmid encoded enzymes. They were 60 or 38 times more active than chloramphenical against a plasmid carrying strain of S. aureus while their activity against a sensitive strain of E. coli was identical to that of chloramphenical. It also should be noted that these two compounds are devoid of

asymmetric carbon atoms and, hence, cannot give rise to a stereospecific attachment to a three-site drug receptor.

The reasons why chloramphenicol produces aplastic anemia in certain patients are poorly understood. This toxic effect is not related to inhibition of protein biosynthesis which with chloramphenicol is totally specific for procaryotic cells. A reasonable hypothesis is that patients with an inborn metabolic capacity reduce the nitro group (perhaps to a hydroxylamine) which then conjugates with an immunogenic macromolecule that in turn immunizes against chloramphenicol as a hapten. Antichloramphenicol antibodies have been evoked by injecting an experimental conjugate. If the mechanism of causing aplastic anemia were dependent upon the aromatic nitro group, chloramphenicol derivatives with strongly electronegative non-nitro substituents should be safe. Thiamphenicol in which the para-nitro group has been replaced by -SO<sub>2</sub>-CH<sub>3</sub> has been clinically tried in Europe, so far without reports of cases of aplastic anemia. The Hammett constants (a measure of relative electronegativity) for p-NO<sub>2</sub> and p-SO<sub>2</sub>-CH<sub>3</sub> are 0.78 and 0.73 respectively.

The preceding capsule analysis had led to the recognition of a prototypical structure which compounds must possess in order to act like chloramphenical. This is shown in Figure 5.

Figure V

#### GENERALIZED STRUCTURE OF CHLORAMPHENICOLS

The first requirement is for an aromatic (carbocyclic or heterocyclic) ring system which is substituted with  $R_1$  a strongly electronegative (and non-ionogenic) substituent such as  $NO_2$ , I, Br,  $SCH_3$ ,  $OCH_3$ ,  $SO_2-CH_3$  etc. The size and geometry of the ring system are not critical.

The second requirement is for a small acyl substituent amide bonded to the amino group of the propane chain. This must be strongly electronegatively substituted  $R_2(\text{Cl}_2,\text{Br}_2,\text{ClBr}$  etc) to facilitate entry into microbial cells. Amino-acyl substituted chloramphenicol is highly active against in vitro cell-free protein biosynthesis but fails as an inhibitor of growth and protein synthesis in intact bacteria.

The third requirement is for a propane chain (not shorter and not longer). The specific requirement for the D-threo conformation results from the presence of two asymmetric carbon atoms in the molecule of chloramphenical. This requirement is not absolute when different substitutions (fig. 4) abolish the two asymmetric centers. The amide hydrogen and the hydrogen on carbon 2 of the propane chain are essential and can not be substituted.

This study has attained a level of completion at which it is possible to transmit the conclusions to a medicinal chemistry laboratory to serve as a blueprint for the synthesis of superior second-generation chloramphenical derivatives. Research studies on the causation of applastic anemia by chloramphenical remain an urgent requirement.

#### Literature Cited:

- 1. BRIDGEN, P.J., G.A.M. CROSS, and J. BRIDGEN. (1976). N-terminal amino acid sequences of variant-specific surface antigens from <a href="Trypanosoma"><u>Trypanosoma</u></a> brucei. Nature 263, 613-614.
- 2. CAMPBELL, D.H., GARVER, J.S., CREMER, N. and SUSSDORF, D.: Method in Immunology, 2 edition, Benjamin, Inc., Reading Mass. 1974.
- 3. GROSS, G.A.M.(1975). Identification, purification and properties of clone-specific glycoprotein antigens constituting the surface coat of <u>Try</u>panosoma brucei. Parasitology 71. 393-417.
- 4. HAHN, F.E. and P.GUNDI : A structural model of the chloramphenical receptor site. Topics in Infectous Diseases  $\underline{1}$ ,245 (1975), Springer, New York-Wien.
- 5. HANSCH, C.,K. NAKOMOTO, M. GORIN, P. DENISEVICH, E.R. GARREIT, S.M. HEMAN-ACKAH and C.H. WON: Structure-Activity Relationship of Chlorampheaicel. J. Med. Chem. 16, 917 (1973).
- 6. HIRUMI, H., J.J. DOYLE, and K., HIRUMI(1977). African trypanosomes;

#### Literature Cited continued:

Cultivation of animal-infective <u>Trypanosoma brucei in vitro</u>. Science 196, 992-994.

- 7. KLEIN, R.S., M.P. KOTICK, K.A. WATANABE and J.J. FOX: Nucleosides LXXIII. Ribosyl analogs of Chloramphenicol. J. Org. Chem. 36, 4113 (1971)
- 8. KOLOSOV, M.N., SHEMIAKIN, A.S. KHOKHLOV and A.I., GUREVICH: Chloramphenicol. In Khimia Antibiotikov I, Iedatsto Adademii Nauk USSR, 1961.
- 9. O'BRIEN, R., OLENICK, J. and HAHN, F. Reactions of quinine, chloroquine, and quinacrine with DNA and their effects on the DNA and RNA polymerase reactions, Proc. Nat. Acad. Sci. U.S.A. (1966) <u>55</u>: 1511-1517.
- 10. TAYLOR, D.W., and G.A.M., CROSS. (1977) The synthesis of a variant specific antigen by <u>Trypanosoma brucei</u> in vitro. Parasitology 74, 47-60.
- 11. VICKERMAN, K. (1969). On the Surface Structure and Flagellar Adhesion in trypanosomes. J. Cell Science 5, 163-193.
- 12. WICKNER, W. and KORNBERG., A. A holoenzyme form of deoxyribonucleic acid polymerase III, J. Biol. Chem. (1974), 249: 6244-6249.

#### **PUBLICATIONS**

- 1. Allison, R.G. and Hahn, F.E.: Changes in superhelical density of closed circular DNA by intercalation of anti-R-plasmid drugs and of primaquine. Antimicr. Agents and Chemother. 11:251-257, (1977).
- 2. Brenner, D.J., G.R. Fanning, A.G. Steigerwalt, M.A. Sodd, and B.P. Doctor. Conservation of Transfer Pibonucleic Acid and 5S Ribonucleic Acid Cistrons in Enterobacteriaceae. <u>J. of Bacteriology</u>, 129:1435-1439. (1977).
- 3. Brenner, D.J., G.R. Fanning, and A.G. Steigerwalt. 1977. Deoxyribonucleic Acid Relatedness Among Erwiniae and other Enterobacteria. II. Corn Stalk Rot Bacterium and Pectobacterium chrysanthemi. Int. J. of Syst. Bacteriol.27,211-221 (1977).
- 4. Fanning, G.R., G. Vaughn, B.:Davis, and D.J. Brenner. Genetic Similarity in Biochemically Atypical Clinical Isolates of Escherichia coli. ASM 77: 61, (1977).
- 5. Hahn, F.E., and Ciak, J.: Bactericidal effects of Combination of ampicillin with anti-R-factor compounds in Salmonella typhimurium R+. Antimicr. Agents and Chemother. 11:176-177, (1977).
- 6. Hahn, F.E., and Ellis, R.L.: Binding to DNA of an antimalarial bis (7-chloro-4-imino-quinoly1) compound, WR 3863. ASM 77: 12, (1977)
- 7. Hahn, F.E.: Experimental elimination of R-plasmids. Proc. 10th Int. Congr. of Chemother. Zurich, Switzerland, (1977).
- 8. Hahn, F.E.; Distamycins and netropsin as inhibitors of RNA and DNA polymerases. Pharmacology and Therapeutics, A 1, 475, 1977.
- 9. B.D.W. Jarvis, MacLean, T.S., Robertson, I.G.C. and G.R. Fanning. Phenetic Similarity and DNA base sequence homology of root nodule bacteria from New Zealand, native legumes and Rhizobium strains from agricultural plants. N.Z.J. of Agriculture Research. 20 235-48 (1977)
- 10. O'Brien, A.D., Thompson, M.R., Cantey, J.R., and Formal, S.B.: Production of a Shigella dysenteriae like toxin by pathogenic Escherichia coli.
- 11. O'Brien, A., Thompson, M.R., Gemski, P., Doctor, B.P. and Formal. S.B.: Biological properties of <u>Shigella flexneri</u> 2A toxin and its serological relationship to <u>Shigella dysenteriae</u> 1 toxin. Infect. and Imm. 15:796-798, (1977).

- 12. Olenick, J.G., Thompson, M.R., and Wolfe, A.D. The inhibitory effect of a shigella toxin on in vitro bacterial protein synthesis. ASM 77: (1977).
- 13. Sodd, M.A.: Analysis of the primary and secondary structure of tRNA. Chemical Rubber Company Handbook of Biochemistry and Molecular Biology, 3rd Edition, Nucleic Acids, Vol. II: 426. Editor: Gerald D. Fasman. Chemical Rubber Company Press, Inc. Cleveland, Ohio. 1976.
- 14. Thompson, M.R., Steinberg, M.S., Gemski, P. Formal, S.B., and Doctor, B.P.: Inhibition of in vitro protein synthesis by Shigella dysenteriae toxin. Biochem. Biophys. Res. Comm. 71:783-788, (1976).
- 15. Thompson, M.R., Dalrymple, J., Gentry, M.K., Formal, S.B., O'Brien, A.D. and Doctor, B.P.: Further Characterization of toxin from Shigella dysenteriae 1. ASM 77: (1977).
- 16. Wolfe, A.D. Influence of cationic triphenylmethand dyes upon DNA polymerization and product hydrolysis by Escherichia coli polymerase I. Biochemistry 16: 30-33. (1977).

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY			1. AGENCY ACCESSIONS			2. DATE OF SUMMARY		REPORT CONTROL SYMBOL			
				OC 6457	1	77 10	01		R&E(AR)636		
3. DATE PREV SUM'RY	4. KIND OF SUMMARY	S. SUMMARY SCTY	& WORK SECURITY	7. REGRA	DING*		P'H INSTR'H	CONTRACTOR		. LEVEL OF SUM	
76 10 01	D. Change	U	U	NA		NL	NL Dires De A WORK		A WORK UNIT		
10. NO./CODES:*	PROGRAM ELEMENT	PROJECT	PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER				
& PRIMARY	61102A	3M16110	2BS01	00 125							
b. CONTRIBUTING						_					
C. CONTRIBUTING	CARDS 114F										
1. TITLE (Procedo with Socurity Classification Code) <sup>©</sup>											
	(U) Military Stress: Circadian and Ultradian Factors										
	12. SCIENTIFIC AND TECHNOLOGICAL AREAS										
016200 Stress Physiology 013400 Psychology											
13. START DATE		14. ESTIMATED COMP	PLETION DATE	IS FUNC	NNG AGENCY			IL PERFORM	ANCE MET	E METHOD	
76 07		CONT		DA			C In-House				
17. CONTRACT/GRANT	. CONTRACT/GRANT NA			10. RES	PRECEDING				IDS (In thousands)		
A DATES/EFFECTIVE:		EXPIRATION:			PRECEDING						
& NUMBER:*	NUMBER:*			FISCAL	CURRENT		5.0 12		20		
C TYPE:		& AMOUNT:					148				
& KIND OF AWARD:		f. CUM. AMT.		70 3.0		140					
19. RESPONSIBLE DOD	PREMIZATION			20. PERI	ORMING ORGA	MIZA	TION				
NAME: Walter Reed Army Institute of Research Walter Reed Army Institute of Resea						f Research					
Division of Neuronsychiatry											
ADDRESS:* Washington, DC 20012			Washington, DC 20012								
RESPONSIBLE INDIVIDUAL			HAME: Hegge, F.W. Ph.D.								
NAME: Rapmund, COL G.			TELEPHONE: 202-547-5521								
TELEPHONE: 202-576-3551			SOCIAL SECURITY ACCOUNT NUMBER:								
21. GENERAL USE			ASSOCIATE INVESTIGATORS								
Constant data 11/2			HAME: Graeber, CPT R.C.								
Foreign intelligence not considered			MAME: Jhorne, D. Ph.D.								
15. REVNORDS (Procedo EACH with Security Classification Code) (U) Stress; (U) Biological Rhythms; (U) Chronobiology;											
(U) Electrophysiology; (U) Performance; (U) Psychophysiology; (U) Human Volunteer						teer					
23 TECHNICAL OBJECTIVE 24 APPROACH, 25 PROGRESS (Purnish Individual paragraphs Identified by number. Procedu text of each with Security Classification Code.)											

- 23. (U) Achievement for an understanding of the temporal organization of biological functions attendent upon sustained exposure to stressors in military environments. Information developed provides indicators of the magnitude and time-course of stressor induced behavioral and physiological disorders that are the precursors of the production of psychiatric and combat casualties.
- 24. (U) Electronic and bioproduct monitoring techniques are employed in the laboratory and in the field to obtain detailed behavioral, electrophysiological, and biochemical measures of functioning during sustained operations. A variety of time series analysis techniques are applied to these data to assess changes that precede and accompany stress responses.
- 25. (U) 76 10 77 09 In collaboration with the United States Army Research Institute of Environmental Medicine, four artillery fire direction center teams have been studied under conditions of simulated sustained combat. Activity, ECG, EEG, and performance speed and accuracy were measured. Two groups were in a single session of 72 hours and two groups in a 38 hr ON 36 hrs OFF 36 hr ON configuration. Neither 72 hr group completed the scenario. Both of the latter groups finished the scenario. Analyses of the physiological and behavioral time series are in progress. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 JUL 76 - 30 SEP 77.

Project 3M161102BS01 MILITARY PSYCHIATRY

Work Unit 125 Military Stress: Circadian and Ultradian Factors

Investigators.

Frederick W. Hegge, Ph.D. Principal:

MAJ Sander Genser, MC; MAJ Daniel Redmond, MC; Associates: CPT R. Curtis Graeber, MSC; John Schrot, Ph.D.

David Thorne, Ph.D., Helen Sing, M.S.; Frederick Weber, M.S.E.E.; SSP Theodore DeWitt.

#### Description

This work unit is directed at the understanding of changes in the temporal organization of biological functions attendent upon sustained exposure to stressors characteristic of military environments. The information developed provides indicators of the magnitude and time course of stressor-induced behavioral and physiological dysfunctions that are the precursors of the production of psychiatric casualties. Work is conducted in both laboratory and field settings to assess the biological costs of sustained performance requirements, rapid translocation across time zones and altered work/rest cycles.

Effort is expended on the development of the technology base required to acquire and analyze the long biological and behavioral data sequences needed to assess baseline, exposure and recovery functions. Non-invasive sensors and recorders designed for field deployment must meet rigorous requirements related to size, weight, ruggedness and reliability. The work involves both the adaptation of measurements previously restricted to the laboratory and clinic, e.g., the electroencephalogram, for use in naturalistic settings, and the development of new measurements that simplify the problems attendent upon field deployment.

#### Progress

#### Studies Made on a Simulated Artillery Fire Direction Center

In collaboration with the United States Army Research Institute of Environmental Medicine (USARIEM), four artillery manual fire direction center (FDC) teams have been studied under conditions of simulated sustained combat. The purpose of the collaboration was to investigate the feasibility of acquiring data on heart rate (ECG), electroencephalogram (EEG) and general activity that could be correlated with individual and small group performance of a well-defined military task.

A light, sturdy cotton vest was developed to serve as an instrumentation platform for two channels of radio telemetry, two EEG preamplifiers and a miniature four-channel analog tape recorder. The vest was well-tolerated by the members of the FDC teams and did not appear to impede their activities as they went about their normal tasks.

The problems encountered in acquiring these data involved electromagnetic noise in the study environment, movement induced artifact in the biological signals, maintenance of electrodes and equipment failure. While these problems are present in any research setting, they are particularly acute in field monitoring. (Note: While this study was conducted in the laboratories of USARIEM, its logistics were those of a field study for WRAIR personnel).

The most difficult signals to acquire with any degree of reliability are the EEG during performance periods. Having extremely low amplitudes, they are most susceptible to noise and motion artifact. EEG electrodes and the paste used to couple the electrodes to the skin caused particular problem with respect to maintaining good contact and low incidence of skin reactions when worn for extended periods of time. Extensive use of EEG recording in the field is not recommended for future field studies. The ECG and general activity measures are, however, robust and high quality recording can be obtained over extended periods of time.

These physiological data are currently under analysis and any conclusions that might be drawn from them would be premature. Preliminary observations related to social and performance factors are discussed in the section entitled "Small Groups and Stress: Studies Made on a Simulated Artillery Fire Direction Center" to be found elsewhere in this volume.

#### 2. Analytic Strategies for Chronobiological Studies

The distinguishing characteristics of chronobiological studies, as opposed to other classes of biological study, are to be found in the frequency with which dependent variable measurements are made and the period of time over which such observations are extended. If one suspects the existence of rhythmicity in biological processes and attempts to define its nature, then sampling interval and sampling duration are largely dictated by mathematical considerations growing out of the hypothesized range of frequencies. The work conducted under the aegis of this work unit is concerned with rhythmicities ranging in period from 24-hours to minutes and consequently data series are composed of many closely spaced measurements extended up to a week in length.

In previous Annual Reports, it has been demonstrated that biological rhythmicities are dynamic rather than static in nature. Amplitudes, frequencies, and phases of rhythms vary in a lawful manner as a function of environmental events and alterations of internal organismic states. The establishment and specification of quantitative relationships between rhythm parameters and stress induced changes in internal states is a necessary step in this research. Time series analysis techniques which operate on an entire ensemble of data are useful in delineating overall frequency structure but fail seriously in their ability to specify changes in frequency structure. This is especially true when the frequencies of interest lie in the circadian ( $\sim$  24 hour) and ultradian (40-120 min) frequency bands.

During the past year, we have completed the adaption of an analytic algorithm known as complex demodulation (CD) for use with biological time series. The analysis produces estimates of amplitude and phase for each data epoch and allows for assessment of the relative contributions of different frequencies at different portions of time series. Intraindividual and interindividual comparisons respecting temporal structure can be made and correlations between temporal structures and environmental events are readily accomplished.

Extensive use of Monte Carlo time series having known properties has been made to investigate the manner in which the CD algorithm operates on data sets. This has permitted a number of technical issues relevant to the analysis of "real" data to be resolved producing a consequent increase in the scope of application of the algorithm. In cooperation with the Departments of Medical Neuroscience and Military Psychiatry, analyses of hormonal excretion, human communication, and social interaction patterns are under way.

#### 3. The Measurement of General Activity: The Actigraph

The difficulties of field monitoring psychophysiological functions were discussed briefly in Section 1. Here we discuss a supplemental strategy for acquiring information from the field that at once bypasses many of the difficulties alluded to earlier and extends our scope of activity. The use of large eyemovements as an indicant of general activity level proved to be a fruitful source of information in our study of the early heroin abstinence syndrome. Eyemovements, measured from the electrooculogram, are subject to all of the difficulties mentioned earlier. Preliminary work indicates that monitoring the frequency of movement of the non-dominant wrist will provide comparable information with significantly less difficulty.

Non-dominant wrist activity is measured with a device called the Actigraph. The size of a pocket match box, the device under development is strapped to the wrist in the manner of a watch and provides minimal impediment to motion and activity. It contains a motion transducer and the electronic circuitry needed to count and store up to one thousand epochs of movement. Depending upon the timing of the epochs, up to a week of data can be recorded and stored. The device will, in its final form, be rugged and hermatically sealed to permit deployment in a variety of settings. It is currently at the final breadboarding stage; the outcome of which will establish the design and permit the manufacture of a miniaturized hybrid version to be undertaken.

Important areas of early application will be in the assessment of work/rest patterns of soldiers on extended maneuvers and before, during, and after long flights. There is mounting evidence that determinations of sleep onset and waking time can be determined with precision from actigraph records. Restless and disturbed sleep are, in principal, also determinable from these records. Release from our dependence upon anecdotal and observational studies of naturalistic sleep will provide information hitherto unavailable.

Project 3M161102B501 MILITARY PSYCHIATRY

Work Unit 125 Military Stress: Circadian and Ultradian Factors

#### **Publications**

- 1. Gatty, R., & Graeber, R.C.: Biorhythms and psychorhythms in attitude research. In Proceedings of the 22nd Annual Conference/1976 of the Advertising Research Foundation, Advertising Research Foundation: New York, pp. 235-252.
- 2. Graeber, R. C., Lubanovic, W., Thompson, M., Halberg, E., Halberg, F., & Levine, H.: Circadian rhythm of performance on a reciprocal tapping task in subjects on a limited free-choice diet. In XII International Conference Proceedings of the International Society for Chronobiology, The Publishing House "Il Ponte": Milan, pp. 39-46, 1977.
- 3. Hegge, F. W.: Complex demodulation analysis of cardiac and activity time series. Proceedings of the 12th Annual Meeting of the Association Advancement of Medical Instrumentation. San Francisco, CA, p. 14, March 13-17, 1977.
- 4. Levine, H., Halberg, F., Bartter, F. C., Delea, C. S., Graeber, R. C., & Jacobs, H. L.: Circadian rhythm in oral temperature on different schedules of drugs and meals, with reference to primary aldosteronism. In Drugs, Biogenic Amines, and Body Temperature (K. E. Cooper, P. Lomax, & E. Schönbaum, Eds.), S. Karger:Basel, pp. 235-241, 1977.
- 5. Levine, H., Halberg, E., Halberg, F., Thompson, M., Graeber, R. C., Thompson, D., & Jacobs, H.: Changes in internal timing of heart rate diastolic blood pressure and certain aspects of physical and mental performance in presumably healthy subjects on different meal schedules. In XII International Conference Proceedings of the International Society for Chronobiology, The Publishing House "Il Ponte": Milan, pp. 139-148, 1977.
- 6. Orr, W. C., Hoffman, H. J., & Hegge, F. W.: The assessment of time dependent changes in human performance. Chronobiol.  $\underline{34}$ , 293-305, 1976.
- 7. Shapiro, A. P., Schwartz, G. E., Ferguson, D. C. E., Redmond, D. P., & Weiss, S. M.: Behavioral methods in the treatment of hypertension A review of their clinical status. Ann. Int. Med. 86: 626-636, 1977.

Project 3E762771A804 Project 3M161102BS01

Work Unit 042 Military Preventive Psychiatry Work Unit 125 Military Stress: Circadian and Ultradian Factors

Investigators.

Principal: COL Harry C. Holloway, MC

Associate: Frederick W. Hegge, Ph.D., David H. Marlowe, Ph.D., MAJ Gregory Belenky, MC, CPT George D. Bishop, MSC, MAJ Robert E. Blaik, MC, MAJ T. Peter Bridge, MC, E5 Joseph Bruer, E5 Stephen Camp, William E. Datel, Ph.D., SSG Theodore DeWitt, Rosemary A. Diliberto, MSW, SFC Jeremiah R. Dixon, Robert N. Dornhart, MA, Joseph E. Fritz, LTC Juan M. Garcia, MC, MAJ Sander G. Genser, MC, MAJ Steven D. Gilbert, MC, CPT Eugene E. Grossman, MSC, Glenn T. Gurley, BA, Stanley Hall, BA, LTC Jesse J. Harris, MSC, SSG Nelson S. Henry, MAJ William Hollinshead, MC, Richard Howard, MA, MAJ Larry H. Ingraham, MSC, CPT John R. Jennings, MSW, John W. Johnson, Jacob Karen, E5 Patricia Kling, George Lampron, Alison Lee, E5 Marie A. McCarty, MAJ David A. McFarling, MC, E5 Patricia Muldrow, Richard J. Oldakowski, MAJ David W. Pearson, MC, MAJ Daniel P. Redmond, MC, Joseph M. Rothberg, Ph.D., CPT Robert J. Schneider, Ph.D., John Schrot, Ph.D., Helen Sing, MS, E5 JoAnne Smith, LTC Albert Tamoush, MC, SSG Charles I. Taylor, David Thorne, Ph.D., Frederick Weber, MSEE

Small Groups of Stress: Studies of Simulated Sustained Operations and Continuous Performance

#### Description

For the past year the Departments of Military Psychiatry and Military Medical Psychophysiology have been engaged in collaborative research with USARIEM to evaluate a simulation utilizing teams from Artillery Fire Direction Centers. During the period of February May 1977, four such teams were observed, studied and evaluated in simulations run at U.S. Army Research Institute of Environmental Medicine. Soldiers in combat routinely must perform complex tasks under conditions of chronic stress of multiple origins. They are often required to perform their tasks continuously and under conditions (such as sleep deprivation and heavy task load) which are known to have profoundly adverse effects on both task performance and individual and social functioning. In order to

evaluate the biomedical implications of current combat doctrine and devise means for the prevention and treatment of neuropsychiatric combat casualties, it is necessary to have a firm data base concerning the effects of multiple stressors on soldiers both as individuals and as members of combat units. Units deserving particular attention are those performing command/control functions. These units perform functions which are both critical to combat operations and known to show degradation under conditions of stress. One such unit is the Field Artillery Fire Direction Center (FDC).

The present research engaged existing FDC teams in simulated combat operations. Although a great deal of research has examined the performance of simplified tasks by individuals under stress, very little work has explored the performance of combat tasks routinely accomplished by teams of individuals. As the use of complex tasks and teams introduces conceptual complexity not easily encompassed by analyses of individual performance of synthetic tasks, it seemed necessary to analyze directly the team performance of complex tasks to obtain generalizable data. It was anticipated that this specific simulation would provide data useful both militarily and scientifically on a number of analytic levels including the operational, biochemical, physiological, psychological and social. If successful, such a simulation would prove to be an invaluable research tool for the study of a number of topics, particularly those concerned with peformance under conditions of stress and continuous operations.

The FDC runs were conducted in the large altitude chamber of USARIEM. Each team consisted of five men; a fire direction officer (FDO), a computer, a horizontal chart operator (HCO), a vertical chart operator (VCO), and a radio-telephone operator (RTO). Two were studied in condition A - following a week of pre-training each team entered the chamber at 0600 Monday morning to attempt a sustained performance operation that they were told might last as long as 82 hours. Two teams were informed that they would be expected to perform for 38 hours in an initial run, followed by 36 hours of recovery time followed by a second 38-hour run.

In each case the experiment was organized by six hour blocks of scenario time regularly broken by a "move" during which psychological tests were administered and equipment was adjusted, maintained and replaced as necessary. Eating military rations generally occurred during this time.

- 1. One team withdrew at the end of 48 hours.
- 2. Two other teams successfully negotiated both  $38\text{-}\mathrm{hour}$  blocks.
- 3. One individual from a team withdrew at the end of the first 6-hour block of the second run.
- 4. The other 82 hour team withdrew from the experiment at the end of 44 hours.

The FDC teams all came from the 82nd Airborne Division. There were differences in experience, time spent in the FDC on the part of individuals and time spent together as teams. Overall, the members of the groups were fairly comparable on demographic variables.

## Progress

- 1. A video and audio archive of all four FDC runs was acquired. This archive is between 80-90% complete with losses in video due to transient equipment failures and losses in audio due to technical problems inherent in the audio system used during the initial two runs. Video quality ranges from poor to fair as a function of the constraints imposed by the size of the chamber, the remote operation of cameras from fixed positions, and the limited number of lenses of various focal lengths available. The individual audio archives recorded on the Honeywell 7600 tape recorder are subject to many of the same limitations as the audio track on the video recording. Both visual and sound quality were particularly poor in recordings of activities in the small chamber during periods of "movement" between blocks of the scenario. This is particularly unfortunate since a fair amount of critical social behavior took place during such break times. The value of the video and audio archives as a source of data has yet to be established.
- 2. Task performance Measures from the WRAIR protocol
  a. Task 1 (Mission latency) and Task 2 (verbalization of error identification).

The data from runs 1 and 2 which were recorded electronically have not yet been retrieved. Retrieval is problematic at this time. The data from runs 3 and 4, recorded on a printed paper tape, have been analyzed and plotted for latency and error.

- b. Tasks 3 and 4 (Group performance measures). These were the responsibility of USARIEM.
- c. Tasks 5,6,7 (video analyses) in the WRAIR Protocol. No formal analyses of the video archive have been performed.

Such analyses are dependent upon the outcome of other preliminary data analyses.

d. Social Process and Communication.

Common patterns were sampled and encoded using paper and pencil recording techniques for runs 1,2, and 4. The data for run 3 were electronically coded and have not yet been recovered for analysis. Recovery is problematical. The other data were recorded using paper and pencil and are being handled in the following manner:

- (1) The first task was the coding and indexing of records completed by behavioral observers. This task was completed on 17 July 1977. Preliminary graphic display of these data was completed on 15 August 1977; this provided a general overview of communication data by groups. Additional time series analyses of these data began 29 September 1977. These will identify recurrent cycles in verbal behavior, as well as provide a basis for comparison with physiological data collected by the Department of Miltiary Medical Psychophysiology.
- (2) The second task was the identification of high and low task loads by reference to video tapes. This task was completed for all four runs on 23 September and allows us to examine the critical area of communication as a function of task load.
  - e. Cerebral hemisphere activation asymmetry.

An attempt was made to acquire EEG data during the course of the first run. Initial equipment failures demonstrated that this was not feasible without equipment modification. No attempts were made to collect EEG data during runs 2 and 3 as per agreement between WRAIR and USARIEM about such use of monitoring equipment. Thus for the period of the first three runs no useful data were collected during task or waking periods. Sleep data were collected. This data are partially analyzed and available under the responsibility of the U.S. Navy. During run 4 EEG data were collected throughout the course of the mission using a redesigned and rebuilt monitoring system. These data have not been fully recovered.

f. Verbal, spatial and handedness testing.

Test data have been tabulated and key-punched and are now ready for analysis.

g. Sleep study.

The initial responsibility is that of the U.S. Navy.

- h. Electro-cardiogram data.
- (1) Medilogger of the data collected, that for runs, 2,3, and 4 are available in the tape archive. These data have not yet been retrieved or synchronized with the main body of archival material. Electronic circuitry and software for retrieval has been developed.
- (2) Analog recording Due to equipment failures and problems that arose in signal telemetry EKG data are not available for runs 1 and 2. Data are available for runs 3 and 4. These data are in the process of being retrieved. Software has been established and tested for creation of an archival record to consist of a time series of inter-beat intervals with associated time and event markings. The software for analyzing this archival record has been developed.
  - i. Non-dominant wrist activity.

Medilogger data are available for runs 2,3, and 4. This data have not been retrieved or synchronized with the remaining archives. An algorithm to calibrate individual movement recorders as well as hardware and software for data retrieved has been developed.

j. Blood chemistry.

Blood samples were collected at the indicated intervals during runs 1 and 4. Analyses of these for serum creatinine phosphokinase, aldolase, electrolyes and cortisol will be done by the WRGH lab within the next two or three weeks. Other biochemical and urinanalysis are the responsibility of USARIEM.

k. Psychological status before runs.

Current status of these data are as follows:

## (1) Questionnaire data

(a) CPI - All CPI questionnaires have been scored and profiles have been worked up for each FDC subject. The profiles for 16 of the subjects appeared to be at least average. Three of the profiles tended to be below average with several scale scores one or more standard deviations below the standardized mean. The final profile had a number of scores markedly below average and resembled a prototypical set of faked scores.

(b) Dogmatism scale - all questionnaires have been scored. Preliminary analysis shows no significant differences among groups.

(c) Rotter Internal-External Locus of Control - All questionnaires have been scored. Preliminary analysis show no significant differences between groups.

(d) Semantic differential scales - have not yet been analyyed.

(e) Personal History Questionnaire - were scrutinized during runs to obtain general information about individual subjects. No statistical analysis has been done or is contemplated at this time.

#### (2) Interview data

Interview data, while providing general information concerning subjects before runs have not yet been analyzed in detail.

1. Mood, activiation and symptom questionnaire analyses are being processed.

#### m. Debriefing interviews.

Each of the four FDC teams was debriefed as a group. These data are available as part of the project archive and provide a qualitative background and context for a number of phenomena under study.

#### n. Performance interviews.

One team was interviewed concerning cognitive deficits experienced during the course of its sustained operations run.

#### o. Behavioral checklist.

One team's behavior was tabulated on an objective checklist of gross behaviors. This data have been plotted and subjected to an initial analysis.

p. In addition to the above, the observer team was regularly debriefed during runs 3 and 4. This debriefing forms a part of the total archive.

## Preliminary Results

Based upon observations made of the groups in the chamber, individual interviews and group debriefing, a number of preliminary statements can be made about the FDC teams, their responses, and the studies in general.

- 1. All of the teams shared certain common perceptions of both the job that they perform and the expected behavior of members of an FDC in the 82nd ABN Division. All saw their jobs as extremely critical in terms of present military doctrine. All viewed themselves as holding one of the, if not the most important, job involved in ground combat. Most agreed with the perception derived from their training that the survival of the division during warfare is completely dependent upon their ability to perform their tasks effectively and quickly. Most also agreed that members of an FDC are under extremely high stress and that the way in which one copes with this stress is to act "crazy", i.e., to engage in a great deal of joking, play, and sophmoric behavior when not actually plotting missions. This is asserted by some as the only way one can survive in the job. No members of the FDCs have ever been in combat. Their job perception is based on their training. The reality and unreality of the scenario was constantly reflected against these training modalities rather than against the legitimacy of the scenario as a simulation of a combat situation.
- 2. Perception of Competence and Task. The four FDCs were divided with respect to their conception of the level of competence that would be required of them in combat and to the level of competence they should strive for. Two groups continuously verbalized a need to be perfect. The training concept of no error greater than 3 mils or 30 meters was one that they attempted to meet at all times. As the experiment progressed, both of these groups began to doubt both the quality and effectiveness of their performance when, in fact, they were performing above ARTEP standards. Both agreed that a 20% error rate, was the maximum allowable in a combat situation. The two other groups did not attempt to maintain the same

standards as the first two groups, but viewed their job as getting fire out with accuracy being taken care of by later adjustments of fire. These two groups, also began to doubt the effectiveness of their performance.

- 3. Response to the Experiment. Overall the response to the experiment on the part of each of the four groups was good. There were expected expressions of annoyance about electrodes, television cameras and monitoring equipment. None of these groups responded to the simulation as if they were actually engaged in battle except for occasional short periods of time, and all were obviously aware that they were in an experiment. There was verbalization throughout the experiment over the need to satisfy the scientists, to provide them with data and much speculation as to what the scientists really wanted. In three of the groups, the expressed contingency for continuing to perform in the face of verbalized fatigue and boredom was the need of the scientists to get their data. After the first day in the chamber, this theme became more common. For open-ended groups the question asked became not "how many hours can we go", but, "how many hours must we go so that the experiment will be a success and the scientists will get their data, and "how long must we go to top other FDCs". These groups also attempted to determine how long the experimenters expected them to go. With the 38 hours groups, continuation was justified in terms such as "Well, we have to go through the same exact things all over again so they can see if we do the same things the second time as we did the first." All of the groups communicated with the observers by posturing the playing to the television cameras, playing with the microphones and attempting to communicate with the observers at the viewing port of the chamber.
- 4. Perception of the Simulation. All of the groups viewed the simulation as an excellent training experience. All felt that it had numerous elements of unreality, although those operating the Command Net and acting the parts of Forward Observers were considered adequately competent. Three groups had extensive experience and rapidly deciphered the task pattern being presented to them. They predicted the kinds of mission sequences and often attempted to predict the kinds of fire adjustment that would be asked for each mission. Each group tended to organize its behavior around the six-hour blocks in which the scenario was written. They divided the time between the scientists time, i.e. time on task in the large chamber, and their time, i.e., time spent in the small chamber during simulated movements. Each group was aware of the structure of its experiments, i.e., whether open-ended run or 38-38-hour runs and attempted to pace themselves accordingly. All groups complained that violence had been done to their modes

of operating in the field. The scientists made most of the decisions that the FDO would normally make, either in training or combat. All groups found confinement in the chamber a source of great discomfort.

Termination of Extended Runs. Neither of the teams in the operation under 86 hour instructional situation completed the scenario to the 72-hour length projected by the experimenters. One run was terminated when a member of the team insisted upon leaving at 48 hours. The FDO requested that the entire team be relieved because "it would be militarily incompetent within three hours." The team members agreed that no member would stay behind once one had seriously decided to withdraw from the experiment. They felt that the integrity of the team depended upon their quitting together. They also agreed that they would quit during a break because quitting during the actual run "would have looked bad on the FDC." None of the individuals who left at 48 hours appeared, to the observers, to be actually at the point of exhaustion. Following their exit from the chamber, several stayed up for four or five more hours discussing the events of the preceding two days. The FDO had stated that if one man left, performance of the remaining four would not be militarily effective. It should be pointed out, however, that for prolonged periods during the run, the work of the team was accomplished by as few as three men.

The other team terminated the open-ended experiment at approximately 44 hours. The FDO, who had not slept at all, had been falling asleep on his feet. The chart operators and RTO, who had had little prior FDC experience, had been making more and more serious errors. The Computer made the basic decision that the team had become ineffective and requested that the officer have them relieved as soon as there was a lull between missions. Upon exiting the chamber there was contention among the group as to whether or not they should go back in. The Chart Operators actually insisted that they would go back in and continue when they thought that the FDO and Computer were going to continue to "attempt to tough it out alone".

The 38-hour groups had no difficulty completing the experiment. One man left of his own volition at the end of six hours of the second run of a "38 hour" team. His reason, given to the investigators, was that, having proved that he could do it once, he had no desire to do it again. His leaving generated a great deal of anger in certain other members of the team. Neither of the 38-hour groups had any performance problems and indeed the third group performed quite well with only four men.

6. Cognitive reaction to chamber. Most of the men indicated that the greatest stressor was confinement in the chamber. Second to this was sleep loss and the repetitive nature of the scenario. Individuals noted physical phenomena, like numbness, headache, coldness, upset stomachs, light headedness and so forth during the latter parts of the runs.

Individual debriefings of the five members of the final FDC team produced a consensus regarding: (1) the increasing difficulty that the men experienced in completing both the verbal and the spatial questions during the course of the long run; and (2) the types of strategies that they adopted to overcome the difficulties. They described having to increasingly reread the verbal passages to be able to answer the questions since the initial reading would leave them with some (but decreasing) memory for individual words or phrases but very little "understanding" of the topic or the passage or the overall flow of information conveyed. As their ability to comprehend and retain the passage decreased they found themselves, in answering the questions, relying more and more on concrete physical matches between: (1) words and phrases in the questions and those in the paragraphs or (2) between specific objects in the questions and those in the paragraphs, and on repetitive scanning of the passages for "key words". The major problem described with completing the spatial questions was an increasing difficulty in either constructing a complete mental three-dimensional representation of each figure or in retaining the complete figure in short term memory while performing the rotations needed to make the required comparisons. The subjects described being unable to mentally complete the figures without having them "disappear" as they started the mental rotation. Strategies used to cope with these problems included: (1) repetitive scanning; (2) mentally labelling specific features or feature combinations of the stimulus figure and attempting to match them with those of the response figures without attempting to visualize the entire three-dimensional object; and (3) counting corners, protrusions or other distinct features of the stimulus object and matching them in number with corresponding features of the response figures.

In summary, it appears as if with increasing sleep deprivation it became subjectively more difficult for the men to encode in and/or retrieve from short term memory both spatial and verbal material. Furthermore, they experienced a decrement in the ability to process a coherent body of verbal information (each paragraph) or spatial information (each figure) in such a way as to derive implications needed to answer questions. The question remains regarding whether these subjective descriptions are confirmed by objective performance deficits and whether any processing deficit is demonstratable independently of memory difficulties. However, it does appear clear that with increasing sleep deprivation the relatively pure spatial and verbal processing strategies initially used by the subjects and seen by them as most efficient could no longer be maintained. Supplementation of the pure strategies with those initially used in processing the other type of information was routinely used by subjects and was seen by them as helpful but not as efficient as the use of the pure visuo-spatial strategies for processing the spatial information and the semantic for the verbal data. Since it is known that the pure verbal and spatial strategies are associated with maximal degrees of asymmetrical hemispheric activation (as measured by the EEG), the switch to mixed strategies with sleep deprivation is consistent with the hypotheses that the reduction of information processing efficiency with fatigue is associated with the conscious inability to regulate patterns of differential hemispheric activity.

8. Group Structure and Organization. Although demographically similar, the four groups were markedly different on other varibles. In some cases, preexisting facets of group structure were reinforced in the course of the simulation. In others, restructuring took place. Each group had formal leaders, the Fire Direction Officer and the Computer. In each group, informal leadership that ranged from taking control of the organization and flow of work to "cheerleading" and the generation of enthusiasm in periods when the group was flagging was observed to develop.

Conflict was observed in all groups. There were arguments, disputes and brief irritated, or angry interchanges between various team members. These were, for the most part, bounded and terminated by the flow of events. The FDOs led their groups quite differently. One FDO team organized activities, took over the work of fatigued members, pushed himself more than he pushed the team, and remained consistently in charge of the group. Another left responsibility for running his group to the Computer and RTO, and spent much of his time clowning, joking, and telling stories.

A third was hard driving, attempting to run all phases of the team's operation, and generated a fair amount of anger within the team. Prior to the run this group exhibited the most conflict, although this was dissipated by the end.

One team was composed of the least experienced personnel. Prior to the experiment, there had been some conflict between the team members and the FDO, of whom they were suspicious.

During the course of the simulation, these men were welded together into a close-knit group that seemed to be marked by mutual respect and appreciation. This was discussed in terms of "we really have gotten to get to know each other up here" particularly in the sleeping quarters and on the trips that the team made together. The FDO of this group tended to lead through the use of "intimate authority" and relied heavily on his Computer.

- 9. Coping with Task Demands. There were differences in the patterns of coping utilized by the teams. Three teams were extremely skilled. Work was well organized and the teams were able to keep ahead of the work and find periods for relaxation and sleep. The other team, however, was quite inexperienced, and devoted almost all of its time to task behavior. When individuals fell asleep, their jobs were taken over by others. That is, one chart operator did the work of two, the FDO would operate a chart, etc. Perhaps the greatest difference in coping and task patterns was found among the various roles on the FDC teams. Those who worked regularly together, the RTO, the computer and the FDO, tended to stay awake, alert and on the job with few lapses into sleep. Those who worked intermittently, e.g., the two chart operators, tended to sleep at every opportunity. The other members of the team had to compensate most often for sleeping chart operators, who either could not be or who were not aroused by other members. In all cases, the teams proved quite able to carry out their missions with losses of personnel to sleep, and when an individual chose to exit the experiment and when another became sick for several hours. In the early morning hours, the job of FDC was often performed by two, or at the most three, people while others slept.
- 10. FDC Core Mission Performance data analysis for two FDC teams has been completed. Team performance was assessed by measuring the frequency of detected internal errors, the frequency of rounds-off-target, and fire mission latencies.

FDC team SOP incorporates redundancy checking procedures for the verbal transmission of fire mission data. Each instance in which a data error was detected by an FDC team member was recorded. Conversely, undetected errors which exceeded artillery standards of accuracy resulted in a round-off-target and were recorded. These measures provided an estimate of team accuracy. An estimate of team efficiency was provided by measuring the elapsed time, or latency, of each fire mission. This time frame began with a call for fire and ended with the transmission of the firing data to the battery.

The data were collected throughout each run and were organized into 5-hour blocks. The data analysis was directed toward finding variations in team accuracy/efficiency related to the presumed increasing stress associated with continuous performance. In addition to the quantitative measures, one team was interviewed after its run to obtain the members' subjective impressions of their performance.

Generally, the core mission results indicate that both teams performed consistently for the duration of their respective runs. Few indications of performance decrement were observed. The accuracy and efficiency of one team's performance showed virtually no change either within or between the two 38-hour runs. The small decreases in accuracy and efficiency that were observed occurred primarily during the final block of another team's 45-hour run. The observed decrements in performance occurred in self-paced, the preparation of preplanned target data, as opposed to task paced mission demands. Furthermore, the decrements were evidenced by moderate changes in latency rather than changes in accuracy.

One team was interviewed approximately 15 hours after the termination of their run. They reported experiencing deficits in attention and short-tern memory as the run progressed. Individual members also reported feeling incapacitated by fatigue and of making perseverative errors toward the end of the run. The result was a verbalization of a perception of rapid deterioration which led to their decision to terminate. The team stopped the run before substantial evidence of deterioration appeared in the performance data.

In sum, the 38-hour run design provided no evidence of performance change. There was suggestive evidence of performance decrement within the 72-hour design but that team terminated its run before substantial changes were observed.

11. A Behavioral Checklist was designed to assist in systemically observing and readily quantifying changes in the general behavior of individual FDC team members. Observers were seated before a bank of four video monitors by which they could clearly see all team members except when the subjects exited into a smaller attached chamber to use the latrine or obtain MCI foodstuffs. A set of headphones connected to the output from individual team microphones permitted continuous monitoring of all verbal communications.

At five-minute intervals the observer noted on a checklist each subject's ongoing behavior at that particular moment. Three major categories of behavior (i.e., whole body posture, current activity, and verbal behavior) were monitored and classified according to a set of mutually exclusive sub-categories for each. The entire data recording process required no more than one minute and proceeded in a fixed sequence beginning with the FDO and ending with the RTO.

Preliminary analysis of the results examined gross behavioral patterns corresponding to different halves of the scenario or its six-hour blocks. With regard to posture, all subjects except the FDO tended to sit whenever they could and sat down more often in the second half of each block. In addition to this variation within blocks, there was an overall increase in frequency of sitting from the start of the scenario until a peak between 0330 and 0600 on the second day (i.e., the midpoint of the total time spent in the chamber) followed by a gradual decrease. The total team frequency of standing or stooping exhibited a rhythmical pattern. Each posture having a peak frequency of occurrence every twelve hours with stopping six hours out of phase with standing. More detailed analyses must be completed before the reliability and significance of these apparent rhythms can be ascertained.

The within block variation in sitting correlates with the activity data in that the subjects were engaged in FDC task related activities more often in the first half of seven of the eight blocks. Obviously, being actively engaged in working is less conducive to sitting.

There was also some consistent differences in the team's behavior between the first and second halves of the scenario. During the first half (i.e., first 22 hours) they worked more frequently, played more frequently, and left the chamber more often than in the second half. Their verbal behavior suggests that they were more relaxed during the first half also since joking, singing, and laughing occurred much more often. As might be expected, they slept more frequently during the second half of the scenario.

The foregoing findings suggest that further examination of the Behavioral Checklist is justified and may lead to a clearer understanding of the time-related changes in the team's behavior preceding their termination of participation. By examining changes in individual behavior it should be possible to determine the influence one subject's behavior may have other another's and whether certain behavior patterns became ritualized over time. Temporal occurrence of food and water ingestion behaviors were also recorded in one run. This data has been tabulated but not analyzed.

RESEARCH	AND TECHNOLOG	Y WORK UNIT S	DAC	DAOC 6454		77 10 01		DD-DR&E(AR)636					
76 10 01	D. Change	S. SUMMARY SCTY	E. WORK SECURITY		IA	NL	CONTRACTO	ACCESS	A WORK UNIT				
O. NO./CODES:*	PROGRAM ELEMENT	PROJECT	NUMBER	TASK A	AREA HUMBER		WORK UN	T NUMBER					
PRIMARY	627710	3E527	71A804		00	047							
. CONTRIBUTING	61102A		102BS01		00		126						
c. CONTRIBUTING	Cards 114F												
1. TITLE (Procede with	Security Classification Cod	n)*											
(U) Milita	ary Psychiatr	y Epidemiol	ogy										
	inical Medici	ne 013400	Psychology	y									
13. START DATE		14. ESTIMATED COM	PLETION DATE	IL FUN	DING AGENCY		16. PERFOR	MANCE MET	нов				
76 07		CONT		DA	1	1	C. In-	-House	se				
7. CONTRACY/GRANT		1		In. RES	OURCES ESTIMA	TE & PROF	ESSIONAL MAN Y	RS & FUN	DS (In thousands)				
A DATES/EFFECTIVE:	N/A	EXPIRATION:			PRECEDING								
b. NUMBER:*				FISCAL	77		4.5		104				
C TYPE:		4 AMOUNT:		YEAR									
		f. CUM. AMT			78		6.5						
E KIND OF AWARD:	ORGANIZATION	T. COM. AMT.	<del></del>	20. PF =	FORMING ORGAN	HOITATION	<del></del>						
				-									
NAME: Walter Reed Army Institute of Research					NAME: Walter Reed Army Institute of Research Division of Neuropsychiatry ADDRESS: Washington, DC 20012  PRINCIPAL INVESTIGATOR (Pumilah SEAN II U.S. Academic Invillation)								
RESPONSIBLE INDIVID				Marlowe, D.H., Ph.D.									
NAME: Rapmun				751.5	TELEPHONE: (301) 427-5360								
				SOCIAL SECURITY ACCOUNT NUMBER:									
TELEPHONE: (202) 576-3551					ASSOCIATE INVESTIGATORS								
21. GENERAL USE					NAME: Datel, W.E., Ph.D.								
Foreign Intelligence Not Considered					NAME: Rothberg, J.M., Ph.D.								
	BACH -It Society Class			NAME	Rothbei	8, J,M.	, FII.D.						
	iology; (U) B	(11)	Military A Dysfunction	djust	ment; (I Psycho-	<ol> <li>Psych</li> <li>Social</li> </ol>	niatric I Factors	11ness	); 				
23. (U) Thi mental fact illness, be	is unit examinates that created avioral dystare giving a	nes militar ate risk fo function an	y organiza or and cond	itiona luce t	l, socia o psychi	al, psyclatric d	chologica lisease,	1, and psycho	l environ somatic				
demographic the psychol psychiatric	e methods of analysis, que logical and se illness and of the soldie	uestionnair ocial scien periods of	e and fiel ces are us	d and	cohort delinea	studies te envi	as well ronments	as me	thods of sk for				
illnesses w way utilizi reporting s selected ra are being of the Army. the Departm syndromes of carried out and other r	10-77 09 Analith a psychology morbidity systems. The term of the carried out on Studies have ment of Militaused by pentereviewing precords analyte disease and	somatic com statistics se material e late 1960 n the analy also been ary Medical etrating wo atterns and sis studies	ponent and and mater s indicate 's. Durin sis of psy initiated Psychophy und and ot incidents are being	d beharials a cong the collar collar ther to sof so deve	available available past ye ric and aborative gy on the raumatic according to the recording	lysfunct e through rise in ear stude health ely between e epide c agents among Ar	ions is agh IPDS in psycholies were problems ween this emiclogy s. Studi	and ot eses are initial of we depart of new es have	ther DA id other lated and omen in rtment an irologica re been Cohort				
prevalence lated illne incidence s cies. For	studies desi esses in Army studies analy technical re July 76 - 30 coors upon on gimelors ap	gned to ass population zing the in	sess the act will be compact of sure Reed 292	ctual carrie ich il Army	distribed out as liness sy Institu	ution of t select yndromes te of Re	f psychol ted Army s on care esearch A	ogical posts	lly re- , as will ng agen-				

Project 3M161102BS01

Work Unit 126 Military Psychiatry Epidemiology

Investigators.

Principal: David H. Marlowe, Ph.D.

Associate: CPT George D. Bishop, MSC, MAJ Robert E. Blaik, MC, MAJ T. Peter Bridge, MC, E5 Joseph Bruer, William E. Datel, Ph.D., Rosemary A. Dilberto, MSW, SFC Jeremiah R. Dixon, Robert N. Dornhart, MA, LTC Juan M. Garcia, MC, MAJ Steven D. Gilbert, MC, CPT Eugene E. MSC, Glenn T. Gurley, BA, LCT Jesse J. Harris, MSC, SSG Nelson S. Henry, MAJ William Hollinshead, MC, Richard Howard, MA, MAJ Larry H. Ingraham, MSC, E5 Patricia Kling, E5 Marie A. McCarty, MAJ David A. McFarling, MC, Richard J. Oldakowski, MAJ David W. Pearson, MC, Joseph M. Rothberg, Ph.D., CPT Robert J. Schneider, MSC, E5 JoAnne Smith, LTC Albert Tamoush, MC, SSG Charles I. Taylor

## Description

The military environment places markedly different and sometimes unique demands and strains upon its populations than do civilian environments. These demands and differences in terms of individual and unit effectiveness and performance, mental and physical health, and behavioral disruption and dysfunction engender chronic affects in peacetime to the Army. In periods of deployment and combat, such stresses may have acute affects on the capability of units to perform their missions. This work unit examines military organizational, social, psychological, and environmental factors that both create risk for and mitigate against psychiatric disease, psychosomatic and physical illness, behavioral dysfunction and disruption of performance as they affect Army personnel and impact on care giving agencies. The methods of epidemiology, including records surveillance, population and demographic cohort studies and methods of the psychological and social sciences are used to delineate factors conducing to risk as well as mitigation for such illnesses, disruptions and dysfunctions.

## 1. The Career Outcome Study

## Description

Urine positivity is assumed to be a risk factor for psychiatric, and physical illnesses as well as behavioral dysfunctions and ineffective performance. This study was designed to assess the long range behavioral implications of urine positivity and its potency as a factor of selection for risk for health and behavioral problems and to evaluate the medical evaluation procedure following designation of an individual as urine positive. A cohort demonstrating this presumed risk factor and controls who entered the Army during 1972-73 was defined in order to prospectively study their individual medical and military careers. The cohorts consist of 1967 individuals with positive urines and 2432 negative urine controls. The rate of matching was slightly over 80% for each cohort.

# Progress

The computer programs used in the analysis of the personnel data (MOS, assignment, and rank) had been written to allow for a maximum of nine changes in those items during the 40 months of the reports. The presence of 10 or more changes was not anticipated and resulted in the generation of an error flag and the deletion of the record from further consideration. Analysis of the third and fourth year of the study has been deferred while the computer programs have been re-written and the cumulative history file re-created to accept the larger number of changes which have been observed in the data. Fortuitously, the 24 month analysis was not affected since the first of the individuals did not accumulate more than nine changes (and consequently appear as an error to be deleted) until the 26th month.

Analysis of the results from the first 24 months has been extended from the material presented in the previous annual report. The material is subject to several interpretations with divergent implications. Considering the urine positive group contrasted with the urine negative group, there are large differences observed in several measures. These differences are seen in the ratio of those who fail to successfully complete two years of active duty (33% versus 22%, the urine positives are 52% worse), in the ratio of annual hospitalizations (322/1000 soldiers versus 234/1000, the urine positives are 41% worse), in the ratio of annual hospital days (7.8 versus 6.6, the urine positives are 18% worse), and in the ratio of the fraction remaining at grade E3 or lower at month 24 (47% versus 35%, the urine positives are 33% worse). An alternative is to consider the unscreened group (which contains the

"naturally occurring" 2% urine positives) in contrast to a urine negative group. The respective ratios change from 52%, 41%, 18% and 33% to 0.9%, 0.8% 0.3% and 0.6% with the unscreened group worse than the urine negative group.

Given that the outcome measures of the urine positive group would remain at the same level with changes in the rate of urine positivity in the population, it is possible to calculate the consequences of adopting one of two extreme strategies with regard to urine screening. We assume for the sake of this argument that we may either urine screen and reject all positives or that we do not do the urine screen at all. For each assumed population urine positive rate, we can project the number rejected as a result of having a positive urine (and consequently, the additional number that must be recruited to compensate), the total number of urine tests that must be administered, and the number of expected early separations which would be avoided. Using the observed career outcome data and an assumed urine positive rate of 10%, we have, for each 1000 soldiers entering basic training, an increased recruiting quota of 111 individuals and the administration of 1111 urine tests assuming the use of the strategy of rejecting anyone with a positive urine. This will give 1000 soldiers entering basic training and 781 successful soldiers by month 24. The other strategy (not to urine screen) leaves the recruiting quota at 1000 (rather than 1111), requires no urine tests (rather than 1111), also starts with 1000 soldiers entering basic training but ends up with 771 successful soldiers at month 24 (rather than 781). The interpretations and implications of the data from the career outcome study are in manuscript for publication in the scientific literature.

2. Psychiatric Hospitalization of Females in the Army

#### Description

This is a four year prospective study which will determine the extent to which psychiatric hospitalization and diagnosis differ between male and female soldiers during the course of their first term enlistment. A further comparison will be made of hospitalization outcome of soldiers receiving traditional basic training versus soldiers receiving sex-integrated basic training. As this study progresses it is anticipated that it will generate hypotheses which can be tested in epidemiological field studies.

#### Progress

Each of the seven basic training posts within CONUS, as a result of a dictate from HQ TRADOC, has submitted rosters of all male and female enlistees entering the Army in the month of June 1977.

In addition, rosters of all male and female soldiers who participated in the Basic Initial Entry Training Test (BIET) at Ft. McClellan, Alabama completed in October, 1976 and male and female basic trainees undergoing traditional training during the same period have also been submitted and received. The investigators are now in the initial process of preparing a case registry which will consist of hospitalization data from the Army Individual Patient Data System (IPDS) and personnel data from the MILPERCEN personnel data from the enlisted master file.

3. The Epidemiology of Suicide in the Army

## Description

In collaboration with the Psychiatry and Neurology Consultant, Directorate of Health Care Operations, Office of the Surgeon General, the total number of completed cases of active duty Army suicides for calendar years 1975 and 1976 were collected. These data have been arrayed and analyzed.

## Progress

The findings of this study are presented in a draft manuscript to be submitted for publication. In brief there were a total of 255 cases during the two-year period, producing a suicide rate of 16.4 per 100,000. The male rate was 16.8, higher than that reported for any of the armed services since World War II. The female rate was 9.9, but is based on only nine cases. The heaviest concentration of male suicides was in the 20-24 age group, which had 103 of the 255 cases, and a rate of 18.3. There were 23 cases at age 23, with a rate of 26.4--the highest specific-year age rate until age 42 was reached. The following ratios obtained between comparison rates: male to female 1.7, enlisted to officer 1.7, white male to black male 1.9. The median length of service for both male and female enlisted suicides was 3.0 years, for male officers 13.8 years. Forty per cent of the entire group was single; 66 per cent of all the cases had no children. Over one-half of the suicides took place at home--family quarters, apartment, parental home or barracks. The predominant method of self destruction was the firearm. June was the highest frequency month, Wednesday day of the week. From a running list of stressful problems extant at the time of suicide, the most frequently occurring untoward circumstance was difficulty of one sort or another with a subject's significant others usually wife, fiance or girlfriend. A five-class taxonomy for categorizing the ascendant dysphoric motivational state precipitating the suicide was suggested from observations and commentary contained in the case files. The results of the stressful problem counts and the taxonomy tabulations can be interpreted as supporting Shneidman's position that suidice is primarily a dyadic phenomenon; that is, one involving two people rather than an individual problem.

## 4. The Expectation Fraction Instrument

## Description

Research carried out in the psycho-social aspects of psychiatric epidemiology during recent years has indicated that availability and quality of an individual's social support networkthat is, those other people from whom an individual will receive reinforcement and aid in coping with life stresses and eventsserves a major function determining the health outcome to that individual as part of the sequel of responses to both acute and chronic stress. Much of the literature of military psychiatry indicates as well that the face to face social supports provided by the soldier's primary group serve in a powerful manner to affect his ability to successfully cope with the multiple stresses of deployment and the battlefield. The Expectation Fraction Instrument represents an initial attempt to develop a quantifiable instrument for assaying and inventorying the self- perceived quality and frequency of contact of an individual with his network of "significant others" - those who comprise his social support system. The successful development of such a model would enable us to relate critical psycho-social processes, the individual's perceived relationship to his social support system, to protection and susceptibility to breakdown while under conditions of stress as well as to the evolution of health outcomes.

## Progress

An initial version of the Expectation Fraction Index has been developed. A pilot study to test its reliability and construct validity is presently underway using members of the WRAIR staff as subjects. It is anticipated that testing and preliminary data analysis will be completed during the first six months of the coming fiscal year.

5. Battle Injuries and Associated Neurologic Disorders Sustained by Active Duty Army Personnel During the Vietnam Conflict

#### Description

This is a collaborative project undertaken by investigators and of this Department and investigators of the Department of Military Medical Psychophysiology. For a complete description and report see Military Psychiatry, Work Unit 039, Military Stress; Health, Performance and Injury Factors.

#### **PUBLICATIONS**

- Blaik, R. "Polypharmacologic drug abuse: presentation and discussion of a case." <u>Journal AOA</u>, Vol 76, 1977, pp. 81-87.
- Collins, J.L., Pearson, D.W. & Wells, J. "A description of the Walter Reed Army Medical Center's in-patient psychiatric service population." Journal of the National Medical Association, Vol 64, No 8, 1073-75, pp. 555-559.
- Datel, W.E. "Source data in military psychiatric epidemiology." Military Medicine, Vol 142, No 61, 1977.
- Rothberg, Joseph M., Holloway, H.C., Nace, E.P., & Meyers, A.L.

  "An application of stepwise discriminant analysis to the characterization of military heroin dependents, illicit drug users, and psychiatric patients." <u>International Journal of of the Addictions II</u>, 1976, pp. 819-830.
- Schneider, R.J. & Rothberg, J.M. "Predicting involvement with illicit drugs: development of the instrument." Educational and Psychological Measurements 36, 1976, pp. 1055-1062.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				DA OC 6451 77 10 01 DD-DR&E(AR)636							
3. DATE PREV SUMPR	4. KIND OF SUMMARY	S. SUMMARY SCTY	S. WORK SECURITY	7. REGRA		D088'H IH		SPECIFIC DA	CCESS	. LEVEL OF SUM	
76 10 01	D. Change	U	U	NA	N	L	(D)		МО	A. WORK UNIT	
10. NO./CODES:*	PROGRAM ELEMENT	PROJECT	T NUMBER	TASK AF	REA NUMBER	-		ORK UNIT N	HUMBER	t	
& PRIMARY	61102A	3M161102B	3501	00		12	7				
b. CONTRIBUTING						_					
c. CONTRIBUTING	CARDS 114F	ļ									
(U) Biologi	cal modulation recently control of the control of t	n of milita			sycholo	ev 0	12600 P	harmac	olog		
76 06		CONT	PLETION DATE	DA	IG AGENCY	1		. In-H			
17. CONTRACT/GRAN					URCES ESTIMA	TE .	PROFESSIONAL	HAL MAN YRS & FUNDS (In		DE (In thousands)	
& DATES/EFFECTIVE	z: N/A	EXPIRATION:			RECEDING		7		1	1,20	
L NUMBER:				PISCAL	77		7		4	139	
C TYPE:		& AMOUNT:		YEAR	78		7		).	55	
& KIND OF AWARD:	O OPGANIZATION	f. CUM. AMT.	<del>-                                    </del>	TO PERF	RMING ORGAN	TATION				22	
RESPONSIBLE INDIVI NAMERAPMUND TELEPHONE (202 21. GENERAL USE Foreign Int		Considered		PRINCIPAL NAME:  PRINCIPAL NAME:  TELEPH  SOCIAL :  ASSOCIAT NAME: H	Washing LINVESTIGAT Tyner, ONE:(202) SECURITY ACC E INVESTIGAT OUTSH, (Ursh, (Uylie, I	LTC 576.	-2139 .R. Ph.D.	0012	ne il fin themp	ance;	
(U) Neuroph	nysiology; (U)	Neuroanato	omy; (U) St	ress							
effects bod physiologic military pe 24. (U) A and respond assessed by potentials; electron mi areas; and tion of exce	Investigations lily responses parameters wh	will seek to stress hich collect  of performs ing and the ogic record ive and exp histochemis modification es or other	to describ and injury ctively def ance will be e role of i ding of int perimental stry; stimu ons of horm r drugs.	the the r, and rine the creation of the creation neuroscillation of the creation of the creati	means he to disc ne optimated using the factor al factor	oy wheren all constant or ing the constant or index call the constant oy ab	ich the those condition he tech n performacell echniquing of dilation	e nervo combina ons for aniques ormance cular b ues of liscret and/or	ous sation reff s of e var pioel lighte br r adm	operant riability lectric at and rain ministra-	
	16 10 - 11 09										

repetitive task may be distinct from the rhythmicity of task accuracy; continued development of animal models for performances demanding choice; extended descriptions of animal performance following brain injury and sensory loss; discovery of cells of brigin of monkey spinocerebellar brain path; discovery of new spinal cord paths

concerning cardiovascular function; discovery of cerebral cortex cells responsive to pain, and sensations from the viscera. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 76 - 30 Sep 77.

Project 3M161102BS01 RESEARCH ON MILITARY DISEASE

Work Unit 127 Biological modulation of military performance

Investigators.

Principal: Tyner, LTC C.F.

Associate: Hursh, CPT S.R., Manning, CPT F.J., Petras, J. Ph.D.;

Wylie, R.M. Ph.D.; Elsmore, T.F. Ph.D.; and

Miller, M.G. Ph.D.

# Description.

Work within this unit directs the technologies of multiple disciplines toward the analysis of biological modulation of military performance. The interaction of biology and behavior can be conceptualized on many levels. At one extreme is the manner in which the biological endowment of the normal individual controls behavioral adjustment to the military environment. This endowment may include how behavior is organized, how it is motivated, how it adapts to changes in environmental conditions, and how it may in fact alter the basic biological constituents of the individual. As a military medical research endeavor we are not only concerned with optimizing behavioral functioning but are also concerned with various so-called disorders of behavioral processes generated by a military environment; that is, why behavior becomes disorganized, unmotivated, maladaptive, or pathogenic. We tend to expect these disorders under conditions which appear stressful, noxious, complex, and demanding, conditions which for a variety of reasons are identified with military combat situations involving coping with life-threatening environmental circumstances, continuous or unusual scheduling of activities, and complex or conflicting task requirements. The first five specific activities described below address questions of how the normal biological endowment predisposes an organism to various forms of behavioral variations and breakdown including extreme cases in which the behavior literally generates physical disease (e.g., gastric ulcer).

At the other extreme are considerations of how alteration of the biological environment of the individual influences the normal structure and function of behavior. In this case we are concerned with the potential traumatic direct effects of a military environment on the nervous system and how that may in term disorder the behavior of the individual. For example, how the loss of normal limb afferents would alter motor functioning. Behavioral adjustment in this case is modulated directly by changes in the biological endowment and indirectly by the traumatic effects of a military situation. The last three activities described below address questions of how disruptions of the biological endowment may generate behavioral changes and what neural systems are important in mediating these disorders.

BIOLOGICAL RHYTHMS AND THE MODULATION OF BASIC BEHAVIORAL PROCESSES.

The experiments covered in this section deal with orderly periodic

fluctuations in behavior (i.e., behavioral rhythms). Recent technological developments have increased the likelihood that military operations in conventional wars will involve essentially continuous round-the-clock operation, or operations at unusual times of the day. While it is well established that performance under such conditions will be subject to rhythmic fluctuations (circadian and ultradian rhythms), little is known of variables affecting such rhythmic processes, and little is known of dependent variables or behavioral processes most likely to show such variations.

What literature does exist on behavioral rhythms deals almost totally with behavioral output, or the rates at which behavior is emitted. The studies to be dealt with in this report are an attempt to investigate rhythms in aspects of behavior that are orthogonal to rate, such as the quality or accuracy of a performance. The following questions are of particular interest: 1) what types of behavior will show qualitative behavioral rhythms? 2) what variables are effective in modulating behavioral rhythms? and 3) what are the relationships (phase, amplitude, modifiability) between quantitative and qualitative aspects of behavioral rhythms? The question of the nature of the control of the rhythms, either by an endogenous biological clock or by exogenous entraining stimuli is not of particular concern to these studies, since it is assumed that the military operations to which the results of these studies may be applied would be carried on in environments containing powerful external entraining stimuli (e.g., day/night cycles). The fundamental strategy in all the experiments to be reported is to train animals to perform a task, then to test their ability to do so at various times of the day and night.

Auditory duration discrimination training in rats: Each of four male albino rats lives in its own experimental chamber containing two levers, a "sonalert" auditory signal generator, a dispenser for delivering 45mg food pellets, a continuously available water bottle, and overhead lights to produce general illumination. Eight experimental sessions are run daily, spaced three hours apart, four with the overhead lights on, and four with them off. Each session consists of a sequence of trials spaced 40 sec apart. Each trial lasts a maximum of 20 sec and is signalled by the onset of an intermittent tone from the sonalert. Each tone burst consists of a train of pulses, one every two seconds, and it is the task of the rat to discriminate the duration of the pulses. If the pulses are short, pressing on the left lever produces a food pellet. If the pulses are long, pressing on the right lever produces food. The difficulty of the discrimination, then, can be varied by altering the relative duration of the short and long pulses. 50% of the trials are of each type. The independent measures of behavior that can be obtained for each session include the overall frequency of responding (percent of trials in which responding occurs), and the accuracy of those responses that do occur. Within this context, a prior experiment in which the difficulty of the task was varied by altering the difference between the long and short tone durations showed that there was indeed a rhythm in both accuracy and percent of trials completed. As the problem was made more difficult, the rhythm in work rate decreased in

amplitude, but the amplitude of the accuracy rhythm remained roughly constant. In a second experiment completed this fiscal year, the rhythm in work rate was modulated by increasing the number of lever presses required to indicate a choice. The analysis of these data is incomplete, but preliminary results suggest that there was a similar dissociation between the modulation of the work rate rhythm and the accuracy rhythm. A final experiment involves a brief evaluation of the role played by regular twelve hour cycle of houselight illumination in the entrainment of the rhythms. Following a stabilization period with the normal 12/12 light cycle, the animals were tested in continuous light for 7 consecutive days. They were then returned to the normal 12/12 cycle for restabilization, then run for 7 days in total darkness followed by return to baseline. Preliminary analysis of the data for the continuous light manipulation indicates that, as expected, the animal's rhythms in work rate disappeared under these circumstances. Further analyses will be undertaken to determine effects on accuracy rhythms, and the relationships between the two rhythms.

Quantitative and qualitative behavioral rhythms in primates: Several experiments are underway investigating variables controlling quantitative and qualitative rhythms in various complex behaviors in Rhesus monkeys.

Microanalysis of fixed-ratio performance. Two monkeys are being run on simple schedules in which one food pellet is provided for every 50 key presses (FR 50). Sessions are run at various times of the day. Extremely fined-grained data collection procedures are being used in order to enable a detailed analysis of performance changes as a function of time of the day under these simple conditions. Preliminary results suggest that response durations may be as sensitive an indicator of rhythm effects as the more traditional measure of time between responses.

Interoceptive stimulus control. When a behavioral response is more likely in the presence of one stimulus than in the presence of others, it is said that stimulus control has been established. The stimuli may be either exteroceptive, (e.g., a light or a sound) or interoceptive, (e.g., a visceral stimulus or a state of deprivation). It has been suggested that behavior controlled by interoceptive stimuli is more subject to disruption by a variety of pharmacological and toxic agents, as well as other environmental perturbations. Time-of-day effects on behavior might therefore be more profound when the behavior is under interoceptive stimulus control than when it is externally controlled. The present experiment establishes conditions for behavior to be interoceptively controlled. Four monkeys live in experimental cages containing two keys. In order to get food, the monkeys must complete at least 10 but not more than 15 presses on the first key (key A) before responding on the second key (key B). Switching to key B either too early or too late results in a 30 second period of time (timeout) in which food is unavailable. The stimulus for switching, therefore, is the completion of the required number of responses with no external cue provided. The behavior of switching therefore, must necessarily be under control of some interoceptive cue. To increase the likelihood that the internal cue

is the number of responses completed rather than the amount of time spent responding, a required delay is imposed between successive responses on key A. Each time the animal presses key A, both keys turn red for a period ranging from 0.1 sec to 0.9 sec. Pressing either key during the delay results in a timeout. For any given run of key A responses, the delay remains the same.

The results of this experiment may be analyzed in several ways, the most promising of which is signal detection theory, which allows separation of the organism's sensitivity to the stimulus dimension controlling behavior from factors affecting the bias for alternative responses. Data thus far indicate that a circadian rhythm exists in the animals' tendency to respond, with little effect on the measure of sensitivity.

Delayed stimulus control. Complexity of behavioral procedures also increases their sensitivity to disruption by a variety of influences. Situations which impose a delay between a signal and the opportunity to emit one of two responses based on the nature of that signal are particularly difficult and sensitive to disruption. The four monkeys in this experiment are required to respond to terminate a train of tone pulses in which the tones are either shorter or longer than 0.6 sec. Following a delay period ranging from 1 to 9 sec, the animal has an opportunity to respond indicating whether the tones in the preceeding pulse train were long or short. Correct choices are followed by food, and incorrect choices, or responses during the delay period are followed by a timeout. The animals in this experiment are still in various stages of training, so little may be said of any rhythm effects.

Acquisition of new behavior. A fourth task has been developed which requires the subject to press three keys in a predetermined sequence of four responses. For example, food may follow a sequence of presses such as key 1, key 2, key 3, key 1. The correct sequence is changed at the start of each session so that the acquisition of the solution can be studied repeatedly. During the early part of each session the subjects typically attempt a variety of different solutions. Any correct response - such as key 1 in the last position of the example above - is signalled as correct regardless of the accuracy of the other three responses, but food only follows entirely correct sequences. The subjects have now achieved a high level of accuracy requiring twenty to forty attempts before solving the sequence problem consistently. A computer system of control and recording has been perfected which permits automatic programming of new problems without experimenter intervention and automatically records the details of each acquisition process. The study is now at the stage of rotating sessions around the clock to sample performance at all hours of the day and night.

PRINCIPLES OF BEHAVIORAL CHANGE. This program of research has continued at a slower pace while computer facilities were implemented in the laboratory to permit more reliable and complete recording of the course of behavioral change. In a manner similar to the previously described experiment, subjects learn a new sequence of responses during each test session. Previous work has shown that visual signals for reward after

fractional portions of the correct sequence greatly facilitated acquisition of the solution. This study has now been published. The next steps will include the following investigations:

(a) manipulation of the length and complexity of the sequence problems to determine the fundamental determiners of problem difficulty.

(b) manipulation of the signals for reward to determine if locus, and duration are determiners of their effect.

(c) manipulation of the amount of food reward for solutions relative to non-solutions to determine how primary motivation influences solution acquisition.

THE BIOLOGICAL ECONOMY CONTROLLING CHOICE BETWEEN DIFFERENT COMMODITIES. Economic factors strongly influence the variability of behavior and the susceptibility of continuous performance to biological rhythms. Several follow-up experiments were conducted similar to those reported previously studying choice in a natural economic system among different commodities. Two monkeys have been studied with choices between two sources of food and one source of water. The new experiments sought to eliminate the level of water deprivation as a variable. Despite large changes in total daily water intake, similar relations described choice. For choices involving the two foods, the subjects prefer the source which provides the most frequent opportunities to eat. For choice between food and water, performance is largely independent of the frequency of opportunities to eat or drink in a given period as long as the total daily ingestion of food and water is held constant. Thus, these experiments confirmed the earlier findings that choice between substitutable or similar commodities is controlled by different variables from those that govern choices between non-substitutable or dissimilar commodities.

The study of choice between electrical brain stimulation (EBS), reinforcement of the lateral hypothalamus and food in rats has been extended to include brain loci which generate stimulus bound eating when continuously stimulated. Despite this additional element, responding for EBS, a nonessential commodity, still declines with reduced opportunities to stimulate while responding for food, an essential commodity, increases with reduced opportunities to eat. These functions parallel what economists call elastic and inelastic demand, respectively. Several rats have been studied with these loci with two additional electrodes in the ventromedial hypothalamus (VMH), the reputed food satiety center. These electrodes will be used to lesion the VMH prior to a replication of the initial study with varied opportunities to eat and self-stimulate. It is proposed that the VMH lesion may alter behavior for food reinforcers to more closely resemble the elastic demand function for lateral hypothalamic brain stimulation. Such a demonstration will help solve the riddle of the relation between brain stimulation and food, and will help to explain the relationship between the VMH and the LH in the control of eating. As a bonus, these studies may help define in physiological terms the economic concept of elasticity.

AN ACTIVITY-STRESS ULCER MODEL. Albino rats were housed in individual cages allowing continuous access to a running wheel, but only one hour's access to food each day. A reliable pattern of changes in activity, food

intake, and body weight ensued, culminating in severe debilitation and, barring intervention, death. Wheel running activity rose slowly for several days, then very steeply for 2-3 days, peaking at levels 100-700% above control (free food) levels, and then declining equally sharply for 2-3 days prior to death. Food intake generally increased moderately over the first few days of this regimen, leveled out at about 125% of initial intake (40-50% of free food control levels), and finally dipped sharply just prior to death. Body weight of course fell steadily during this procedure, with most animals losing 20-40% altogether. These patterns were so reliable that, after the first series of subjects, we were able to use the dip in food intake to below 2.0 g as a sign that death was imminent in a given rat, and thus take measures to prevent both unnecessary suffering and preserve tissue from autolysis (i.e., barbiturate overdose and rapid dissection). Control rats given either one hr food access, or continuous running wheel access, but not both, remained healthy, both "clinically" and at microscopic level (described below). Body weight and/ or age were potent determinants however. For young rats weighing 150-175g, 85% of all subjects were sacrificed within 2 weeks. Within the 150-225g range, the correlation between starting weight and days until sacrifice was 0.79. Sex of the subjects was not an important influence independently of weight (female rats weigh less than age-matched males).

Microscopic analysis of activity-stress lesions. Gastric lesions produced by the activity-stress were evaluated by light-microscopy. The glandular portion of the stomachs of rats sacrificed under the conditions described above all showed extensive pathology, ranging from mucosal hemorrhage, with or without focal necrosis, through acute ulcers characterized by discontinuity of the muscularis mucosa. All rats showed the entire range of pathology. In addition, 33% had at least one lesion showing signs of regeneration. Focal erosion predominated, but all experimantal rats had at least one ulcer penetrating the muscularis mucosa. No quantitative or qualitative relations between stomach pathology and survival probability or duration could be uncovered. Besides being a highly reliable, easily accomplished technique for induction of gastric pathology, neither equipment nor labor intensive, this activity-stress technique is apparently the first rodent "ulcer" model to reliable produce classical ulcers (penetration of the muscularis mucosa layer). Its a priori innocuousness, and the apparent lack of correlation between stomach pathology and survival, are two obvious lines of pursuit.

HORMONAL MODULATION OF DRUG EFFECTS: The past year's work in this area constituted a continuation of studies investigating the effects of representative drugs on the operant behavior of adult female albino rats. Specifically, the cyclic changes in brain norepinephrine, dopamine, and serotonin corresponding to the rat's 4-day estrus cycle suggested that both behavior and drug/behavior interactions dependent on these systems should vary with the estrus (i.e., hormonal state of the subject). Estrus state was assessed by daily vaginal smears, and lever pressing was reinforced with food on intermittent schedules. Half the subjects were trained under a variable-interval (VI) contingency, encouraging high rates of pressing (1000-5000/hr). The remaining rats were reinforced only for responses separated by 30 sec or more (a DRL-30

schedule). In Experiment One, the rats were put in the experimental chambers for only one hr each day, and were fed only enough after each day's session to maintain body weight at 85% of free-feeding weight. After performance stabilized, the effects of saline, amphetamine, and pentobarbital were assessed. All injections were given i.p. 15 min before the scheduled start of a session. Amphetamine sulfate (A) doses were 0.5, 1.0, and 2.0 mg/kg; sodium pentobarbital (P) doses were 5, 10, 20 mg/kg. Each rat received each dose of each drug twice, once while in estrus (90% or more cornificed epithelial cells in smear taken at 1530 hrs), and once while in diestrus 90% normal epithelial cells); saline injections were given twice in each of the hormonal conditions. Findings suggested both drug and drug by schedule/behavior effects, but no effect of hormonal state. That is, A appeared to elevate the low rates of pressing induced by the DRL schedule, but depress the high rates induced by the VI schedule. P tended to elevate both high and low rates of responding at 5 mg/kg, and depress both high and low rates at 20 mg/kg, with the intermediate dose producing equivocal findings. These conclusions were essentially independent of the estrus/diestrus distinction. A similar experiment, with new subjects, employed not 1-hr, but 6-hr, testing sessions. These were conducted automatically, between 1700 and 2300 nightly, the first 6 hrs of the dark phase of the light/dark cycle in the animals' quarters, and also the period of peak sexual receptivity on the estrus day of the cycle. Drug effects were similar to those of Experiment One, but intensified and with far less intersubject variability. In addition, there was a suggestion, but only a suggestion, that drugs administered on estrus days produced a slightly larger and longer lasting effect. This is somewhat surprising if real, since the direction and extent of the drug effects was so dependent upon both the dose level and the baseline behavior (reinforcement schedule?). The project was discontinued in June due to the reassignment of the investigator.

RECOVERY OF MOTOR FUNCTION AFTER LIMB DEAFFERENTATION (DORSAL RHIZOTOMY). Abolition of sensory inputs from a single upper extremity of a monkey by surgically cutting the dorsal roots innervating the limb results in a profound motor deficit. Without explicit experimental intervention the animal does not use the limb in even the most rudimentary manner; in ambulation and climbing the animal uses only the three normal limbs; the animal does not use the deafferented limb to take food or in defensive or aggressive behaviors.

Nevertheless, by use of operant techniques, the animal can be trained to perform specific tasks with the limb, and it has been shown that restraint of the normal upper extremity for several days elicits return of function of the deafferented limb which persists after removal of the restraint.

The demonstration that recovery of function of a deafferented limb can be induced by appropriate experimental intervention has challenged two major views about the organization of the nervous system. One is the view that control of movement is maintained only by the successive elicitation of reflexes. The second view is that conditioning of movement depends upon sensory feedback from the active muscles and limbs. The response to the challenge offered by the demonstration of recovery of

function after dorsal rhizotomy has varied from insistence that the challenged theories are true and therefore some residual sensory input from the limb must be spared by dorsal rhizotomy, to acceptance of the results and a new emphasis on the alternative views that the central nervous system plays a dominant role in the organization of movement, and that successful motor programs can be conditioned in the absence of sensory feedback from the active muscles and limb.

The argument that recovery of function after dorsal rhizotomy depends upon sparing of residual sensory inputs has recently been strengthened by reports of a large number of unmyelinated afferents in at least some lumbar ventral roots in the cat, and that at least 5% of dorsal root ganglion cells associated with myelinated fibers have central processes entering the spinal cord via the ventral roots. These potential sources of sensory information would be spared by dorsal rhizotomy which leaves the dorsal root ganglion intact. In spite of these reports, studies have thus far failed to find sensory function in the deafferented limb.

The present study was designed to determine to what extent the recovery of function after deafferentation depends upon risidual sensory inputs. After being trained to flex the forearm at the elbow through an angle that is equal to about one half the full excursion allowed in the restraining apparatus used, both normal and deafferented monkeys are tested by requiring them to perform the same task with varying masses attached to the forearm restraint. Successful performance consists of performing at least the criterion flexion and is rewarded with a small amount of liquid nutrient. The criterion flexion raised the mass against gravity a distance of about 16.5 cm. The essential strategy of the experiment has been to use a task which yields a sufficiently high rate of reinforcement to maintain repetitive performance throughout an hour and a half session. The variability used to describe lifts are acceleration velocity and position. The presence or absence of sensory inputs from the responding limb should be reflected in the degree to which the performance of the normal and deafferented animals tends to be the same or different. At this time, three normal animals and one unilaterally deafferented animal have been tested on a range of different weight. A second deafferented animal has been prepared and is currently undergoing preliminary testing in the task with no external mass.

When the load was different in each session both the normal animals and the deafferented animal reached a maximum height independent of the load. The rate of success of the deafferented animal was within the range achieved by the three normal animals and was also independent of load. Furthermore, the deafferented animal lifted the unknown load to the criterion level on the first lift of the session. Measurement of acceleration revealed that the early part of the lift was load dependent even though the final height was load independent. At the lightest load acceleration rose rapidly to a peak whereas at heavy loads initial accleration was low and the peak of the lift was achieved only by one or more discrete increments of force which appear as discrete and abrupt changes in accleration. On the first lift of a session, the normal animals rarely reach criterion and are not directly comparable to the deafferented animals for that reason.

When the animals were tested by changing the loads within in session at 5 minute intervals, the ability of the deafferented animal to compensate broke down, and all variables describing the movement showed an increased relationship to the load, including the per cent success. In contrast, although in these trials the first lifts at a new load of the normal animals were inversely related to the load, within a few lifts the normal animals showed complete compensation on all variables.

The deafferented animal has also been unable to avoid the consequences of muscle fatigue induced by heavier loads when the load is varied each day. When the same load is repeated each day, all animals distribute their responses through the session in a pattern of runs and pauses. Although normal animals change this pattern when the load is changed, the deafferented animal, when run daily with the heaviest load in the series, required 16 weeks to establish a stable pattern of response. When the loads were changed daily, the variance in the pattern increased, decreasing again to a stable pattern only when he was again run day after day at the heaviest load.

At present it is difficult to assess the role of proprioceptive feedback pathways in the performance of the normal animals. Because the first lift of an unknown weight is load dependent, the gain in feedback pathways must be low. Once the normal animal has executed a lift we must assume he has the information required to either generate central commands appropriete for the load or to appropriately adjust the gain in proprioceptive feedback pathways or both. The deafferented animal clearly lacks proprioceptive feedback pathways with loop delays of less than about 100 msec because even when he achieves the criterion height on the first lift independent of load, this is done only by executing discrete corrections in the trajectory at intervals longer than 100 msec. That his performance does not depend upon any residual sensory inputs is indicated by his inability to achieve independence of load during trials of 5 minutes when he has executed 30 to 100 lifts.

These results further indicate that normal animals receive sensory information related to the relative state of muscular fatigue and that the deafferented animal lacks these sensory inputs. The distribution of behavior in the normal animals is directly related to the load in a way that allows the animals to avoid a state of muscle fatigue which would impair performance. On the other hand, the deafferented animal requires experience in a number of sessions to develop a pattern of response which enables him to avoid load dependent errors. It is not entirely clear what contributes to the acquisition and maintains this behavior although the density of reinforcement clearly plays an important role in at least the initial acquisition. Observation indicates that the deafferented animal works at rates which generate heavy breathing, unlike the normal animals, and the distribution of responding may be related to respiratory factors.

It is difficult at present to assess to what degree the deafferented animal regulates the variables associated with an individual lift. One difficulty is that we can only indirectly infer the nature of the command signals from observation of the variable describing the lift. That some degree of regulation of an individual lift is involved can be concluded

from the observation of discrete corrections observed in the record of acceleration on initial lifts but not observed late in a session when acceleration has become independent of load. Further evidence of regulation is indicated by analysis of data which indicate that the time during which a net upward force is applied to the load is inversely related to acceleration. Lifts of heavier loads, which are associated with lower initial accelerations, are prolonged with the consequence that position, the second integral of acceleration is less dependent upon load than the initial values of acceleration.

A factor which may contribute to the appearance of regulation is indicated by our preliminary recordings of electromyograms of the major antagonists of the elbow. These recordings suggest that there is strong co-activation of the two antagonists, biceps and triceps at the start of movement. Coactivation of these antagonists would increase the apparent mass of the limb and reduce the relative effect of external test loads on the variables describing the movement. Consequently, coactivation of the antagonists would generate the appearance of compensation without there being any change in the motoneuronal activity required to lift the different test loads. We do not yet have recordings from the muscles of normal animals so do not know whether coactivation of antagonists occurs in normal animals.

Related studies. A technique for selective destruction of sensory components of the trigeminal nerve in rats has been developed, and the effects of such surgery on feeding and drinking behavior observed. Following loss of oral-facial input, rats remain able to bite, chew, and swallow; they show disturbances in coordination and exploratory behavior, however, and are unable to maintain normal body weight, the degree of deficit being roughly related to the extent of deafferentation. Some recovery of function has been observed, but only after periods of many weeks.

SINGLE-NEURON RECORDINGS OF CAT SOMATIC MOTOR CORTEX. Single-neuron recordings in cat somatic motor cortex have shown a well-defined neuron population sensitive to stimulation of the splanchnic nerves in the abdomen; the cells in question are believed closely related to control of somatic muscles. These results suggest an intimate relation, in identifiable brain cells, between visceral sensation (particularly concerning the cardiovascular system) and the 'voluntary' movement control system.

Additional single-neuron study of cat somatic motor cortex has revealed a significant, new, type of neuron: a cell which appears to receive potent inhibition following skin stimuli sufficiently intense to be painful; these cortical neurons are also believed closely related to control of somatic muscles. The results suggest the existence of a cellular mechanism by which 'voluntary' or 'exploratory' movements might be temporarily slowed or stopped by painful stimuli.

## ANATOMICAL STUDIES OF SPINAL PATHWAYS.

The origin of spinocerebellar pathways. The origin of spinocerebellar pathways was studied experimentally in neonatal dogs and in rhesus monkeys.

Cerebellar projecting neurons were found in all regions of the spinal cord and included the <u>nucleus cervicalis centralis</u>, <u>nucleus centrobasalis</u>, <u>n. dorsalis</u>, <u>n. pericornualis ventralis</u>, and additional cell groups in the sacrococcygeal segments of the spinal cord (Petras and Cummings, 1975; Cummings and Petras, 1977; Petras and Cummings, 1977; Petras, 1977). The data suggests that: (1) the central cervical nucleus is an important proprioceptive cell group for neck afferents; (2) the large centrobasilar neurons are the cells of origin for the rostro spinocerebellar tract of Oscarsson and Uddenberg (1964); (3) that the n. pericornualis ventralis neurons project axons to the ventro spinocerebellar tract; and (4) that the giant and medium-sized cells of Clarke's column (the nucleus dorsalis) contribute to the formation of the dorsal spinocerebellar tract.

An intraspinal sympathetic preganglionic pathway; anatomic evidence in the dog. Horseradish peroxidase was injected into thoracic and lumbar paravertebral sympathetic ganglia which have been isolated from the sympathetic chain but whose rami communicantes have been preserved. Labeled reaction product was found many segments rostral and caudal to the level of injection, indicating the existence of an intraspinal sympathetic preganglionic pathway. Following thoracic ganglia injections labeled neurons were found only ipsilaterally, whereas after lumbar ganglia injections labeled cells were noted bilaterally; these findings indicate the existance of both an ipsilaterally and contralaterally projecting intraspinal sympathetic preganglionic pathway.

Project 3M161102BS01 RESEARCH ON MILITARY DISEASE

Work Unit 127 Biological modulation of military performance

## Literature cited.

## Publications:

- 1. Cummings, J.F. and J.M. Petras. The origin of spinocerebellar pathways. I. The nucleus cervicalis centralis of the cranial cervical spinal cord. J. Comp. Neurol., <u>173</u>:655-692, 1977.
- 2. Elsmore, T.F. The role of reinforcement loss in tolerance to chronic delta-9-tetrahydrocannabinol effects on operant behavior of rhesus monkeys. Pharmacol. Biochem. & Behav. 5:123-128, 1976.
- 3. Elsmore, T.F. Time-of-day clocks and SKED: techniques and applications. Behav. Res. Methods & Instr., 9:231-232, 1977.
- 4. Hursh, S.R. The conditioned reinforcement of repeated acquisition. J. Expt. Anal. of Behav., 27:315-326, 1977.
- 5. Hursh, S.R., and Natelson, B.H. Operant schedules dissociate the reinforcing values of lateral hypothalamic self stimulation and food. Neurosci. Abstr., Vol. II, 1976.
- 6. Manning, F.J. Chronic  $\Delta$ -9-THC: transient and lasting effects on avoidance behavior. Pharmacol. Biochem. & Behav.,  $\frac{1}{2}$ :17-21, 1976.
- 7. Manning, F.J. Role of experience in acquisition and loss of tolerance to the effect of  $\Delta$ -9-THC on spaced responding. Pharmacol. Biochem. & Behav., 5:269-273, 1976.
- 8. Manning, F.J., and Jackson, M.C. Jr. Interactions and independence in multiple Sidman avoidance schedules: learned helplessness online? Bull. Psychonomic Soc.,  $\underline{8}$ :257, 1976.
- 9. Manning, F.J., and Mishkin, M. Further evidence for dissociation of visual deficits following partial inferior frontal lesions in monkeys. Neurosci. Abstr., 2:1126, 1976.
- 10. Manning, F.J., and Jackson, M.C., Jr. Enduring effects of morphine pellets revealed by conditioned taste aversion. Psychopharm., 51:279-283, 1977.
- 11. Miller, M.G. The effect of trigeminal deafferentation on food and water intake in the rat. Neurosci. Abstr., 1977.

- 12. Petras, J.M. Comparative anatomy of the tetrapod spinal cord: dorsal root connections. In R.B. Masterton, M.E. Bitterman, C.B.G. Campbell and N. Hotten, Eds. Evolution of Brain and Behavior in Vertebrates, Vol 1, Erlbaum Assoc. Inc: Hillsdale, N.J., 1976.
- 13. Petras, J.M. Spinocerebellar neurons in the rhesus monkey. Brain Research, 130:146-151, 1977.
- 14. Petras, J.M., and Cummings, J.F. The origin of spinocerebellar pathways. II. The nucleus centrobasalis of the cervical enlargement and the nucleus dorsalis of the thoracolumbar spinal cord. J. Comp. Neur., 173:693-716, 1977.
- 15. Pryzbylik, A., Martin, G.E., and Spector, N.H. A method for the continuous measurement of core temperature in small animals. Ann. Biomed. Engineer., 5:122-129, 1977.
- 16. Ribas, J.L. The rat epithalamus. I. Correlative scanning-transmission electron microscopy of supraependymal nerves. Cell & Tissue Res., 182:1-16, 1977.
- 17. Ribas, J.L. Morphological evidence for a possible functional role of supraependymal nerves on ependyma. Brain Research, <u>125</u>:362-368, 1977.
- 18. Tyner, C.F., and Miller, M.G. Selective inhibition of some wide-field sensorimotor cortex neurons by high-intensity skin stimuli. Neurosci. Abstr., 1977.

RESEARCH	Y WORK UNIT SUMMARY		DA OC 6449		77 10 01		DD-DR&E(AR)636						
1 DATE PREV SUMPRY	4. KIND OF SUMMARY	S. SUMMARY SCTY	7. REGR				PECIFIC DA	CCESS	. LEVEL OF SUM				
76 10 01	D. Change	UU	NA		NA		YES 🗆		A WORK UNIT				
10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER 3M161102B S01	TASK	AREA NUMBE		128	ORK UNIT	NUMBER					
b. CONTRIBUTING	OTTOZA	341011028 801	100		-	120							
DEX.OCDE.200000000000000	CARDS 114F												
	Security Classification Code	,,•											
		e to Military Stress											
	CHNOLOGICAL AREAS												
012900 Phys.	00 Psychology   16. Performance method												
		14. ESTIMATED COMPLETION DATE		DING AGENCY					нов				
76 07		CONT	DA			<del></del>	n-Hous						
& DATES/EFFECTIVE:	NΛ	EXPIRATION:	IG. RES	PRECEDING	ATE	& PROFESSIONAL	MAN YRS	L FUN	IDS (In thousands)				
b. HUMBER:*	MA.		FISCAL	77		14		1	430				
C TYPE:		4 AMOUNT:	YEAR	YEAR CURRENT									
& KIND OF AWARD:		f. CUM. AMT.		78 3				309					
19. RESPONSIBLE DOD	ORGANIZATION		20. PER	FORMING ORGA	MIZA	TION							
The second secon		titute of Research				d Army Ins		e of	Research				
Washi	ngton, D.C. 2	0012	Div of Neuropsychiatry										
AUUNESS:			ADDRES	Washir	igt	on, D.C. 20012							
			PRINCIP	AL INVESTIGA	TOB (	Fumial SPAN II II C	Academic In						
RESPONSIBLE INDIVID	NAME:	Meyerh	of	f, JL, MD	Academic In	************							
NAME: Rapmun	NAME: Rapmund COL G				TELEPHONE: (202) 576-3559								
TELEPHONE: (202	SOCIAL SECURITY ACCOUNT NUMBER:												
Foreign Intelligence Not Considered				TE INVESTIGAT									
Foreign inc	MAME: Kant GJ MAME: Belenky MAJ G												
22. KEYWORDS (Procedo	HAME. Belenky MAJ G  22 KEYBORDS (Procedo EACH with Security Closel Eacher Code) (U) stress (U) cyclic nucleotides (U) neurotransmitters								nemittere				
		cerebral injury (U)					neur	our a	IISMI COCI S				
		PROGRESS (Furnish Individual personals Ide					Classificati	en Code.					
studies and	recommendati	stress, providing dat ons for prevention an	d/or	treatme	nt	of breakd	lown in	1110	ldiers				
To examine neurochemical mechanisms mediating sequelae to penetrating cerebral injury, providing recommendations for treatment of soldiers.									0 0 ,				
							ac h	- <b></b>	no?				
		le of neurotransmitte											
response to stress. Effect of stimulation or lesion of specific pathways (i.e., noradrenergic, dopaminergic, serotonergic). Effect of stress or centrally-acting													
hormones on cyclic nucleotides and neurotransmitters in specific brain regions. In- vivo determination permitted by use of microwave enzyme inactivation system designed in													
this laboratory. Effect of penetrating cerebral injury on regional neurochemistry													
(i.e., cyclic nucleotides, gamma aminobutyric acid, glutamic acid, acetylcholine, amino													
acids, norepinephrine, dopamine and serotonin) and on seizure threshold.													
25. (U) 76 10 - 77 09 Neurochemical responses have been measured simultaneously													
		iety of stressful con											
according t	o type of str	essor. Cold stress e	eleva	tes cycl	lic	guanosine	3'5'	mon	ophosphate				
		ain regions examined.											
	cerebellum, brainstem and midbrain. These elevations were not affected by depletion of												
		tions from the locus											
		s norepinephrine. In											
	although a potent stressor, fails to elevate cGMP. For technical report see Walter												
Reed Army Institute of Research Annual Progress Report, 1 Jul 76 - 30 Sep 77.													
	040												

Project 3M161102BS01 RESEARCH ON MILITARY DISEASE

Work Unit 128 Mechanism of Response to Military Stress

Investigators.

Principal: James L. Meyerhoff, M.D.

Associate: Robert H. Lenox, M.D., MAJ, MC; Gregory L. Belenky, M.D.,

MAJ, MC; G. J. Kant, Ph.D; E.H. Mougey, M.S; D.R. Collins,

B.S., L.L. Pennington, B.S.

# I. Mechanism of Neuroendocrine Response to Stress

In order to study neurochemical mechanisms determining adaptive hormonal response to environmental stress, we have successfully developed new models. It is well established that stress causes elevations in plasma corticosterone (1-5) and prolactin (5) while lowering plasma growth hormone (3,4). Corticosterone levels following stress vary with time of day, reflecting the circadian rhythm in testing levels (2). It has been demonstrated that habituation occurs to the corticosterone response induced by handling (3,4). Stress is reported to activate central noradrenergic (NE), dopaminergic (DA, and serotonergic (5HT) neurons (6-12). It has been recently reported that cold stress elevates cerebellar cyclic GMP in rats (13) and mice (14). Cyclic nucleotides are known to activate protein kinases. Rodknight (15) notes a possible stress component in brain protein kinase activity. Increases in cAMP levels in brain tissues have been demonstrated following exposure to NE, DA or 5HT in vitro (16,17) and to NE (but only minimally to DA) in vivo (18).

The foregoing has led us to predict that psychological stressors will elevate cGMP and/or cAMP. An ACTH fragment reportedly lowers brain GABA (19). As ACTH is released in stress and GABA administration lowers cGMP (20), it would seem highly desirable to study the effects of stress (as well as ACTH and other peptides) on brain cGMP and GABA, as well as norepinephrine and cAMP. A method has been established in our laboratory which permits assay of gamma-aminobutyric acid (GABA), glutamic acid (GLU), cyclic adenosine 3'5' monophosphate (cAMP), cyclic guanosine 3'5' monophosphate (cGMP) and norepinephrine in the same sample of brain tissue after microwave inactivation of enzymes, thereby increasing the amount of information obtainable from a single experiment. The assays employed are the radioimmunoassay of Steiner for cyclic nucleotides (21,22) and the enzymatic method of Graham and Aprison for GABA and GLU (23). To ensure optimal support of the stress studies, we have modified assay procedures to increase the sensitivity of our assay system by acetylating our samples as described by Harper and Brooker (J. of Cyclic Nucleotide Research 1:207-218, 1975). This has increased our sensitivity into the femtomole range. It is thought that cGMP is responsive to cholinergic transmission (24,27) and under various conditions, brain tissue cAMP is stimulated by norepinephrine, dopamine, serotonin and histamine (25-27). Further studies in our laboratory have demonstrated that the technique of using

high-intensity microwave irradiation for enzyme inactivation is indispensable for determining levels of cAMP, cGMP and GABA in brain regions (28,29). The elimination of artifact has permitted accurate assessment of levels of these substances in the regions studied. In addition, for many of the regions, the work is unique in that levels have never previously been reported.

A new area of critical importance to understanding the effects of stress on emotional breakdown is being opened up by studies indicating direct effects of steroid and peptide hormones on brain. Corticosteroids bind preferentially to specific regions in brain, notably brainstem and hippocampus (30,32). The binding is both to cytosol and to nuclei and is influenced by level of circulating corticoids (30). Corticosterone administration causes a marked increase in the activity of tryptophan hydroxylase (the enzyme that synthesizes serotonin) in midbrain (32). These observations have led to speculation that the serotonin neurons which project from the raphe cells to the hippocampus (33,34) might mediate the feedback inhibitory effect of glucoccrticoids on the pituitaryadrenal axis. There is controversy over whether or not glucocorticoids depress the single unit firing from raphe cells (35,36). An increase in hippocampal theta is reported to occur 1 hr following glucocorticoid administration (37). Raphe stimulation, which releases 5HT (38), blocks habituation to auditory startle response (39). In one study of glial tumor cells in culture, glucocorticoids doubled the cAMP response to NE but at least a 20 hr exposure to the glucocorticoids was required for minimal effect (40). From the above observation, it appears likely that the direct effect of glucocorticoids on brain is more related to chronic, rather than acute effects of stress.

It has been reported (31) that adrenal cortical hormones facilitate the activity of brain adenylate cyclase in vitro. Activity is decreased following adrenal ectomy and this decrease is reversed by maintenance with dexamethasone or aldosterone. Activity is also decreased by hypophysectomy and this decrease is reversed by either ACTH or dexamethasone. Manipulations which affect adenylate cyclase activity in vitro are very dependent on specifics of tissue preparation and may not alter cAMP levels in vivo. Hence the effect of endocrine manipulations on cAMP levels must be studied in vivo. We propose to carry out such studies and to study the effect of endocrine manipulations on the cold-induced elevation of cGMP as well.

We wished to examine the generality of the observed cGMP elevations following exposure to cold. Since immobilization is a potent stressor (42), we compared the responses of brain cGMP and plasma hormones to immobilization or cold exposure. Male albino rats, WRC strain, weighing between 250-300 gm, were maintained in a light-cycled chamber and habituated to both handling and placement into a plexiglass cylinder twice a day for 1 week. On the day of sacrifice, animals were placed into one of three groups. The first group was placed in the plexiglass holder and

immediately sacrificed by a high power microwave irradiation system, modified in our laboratory (43). The second group was immobilized for 5 minutes in the plexiglass holder, before irradiation. The third group was exposed to cold (4°C, with fur wetted) for 5 minutes and then irradiated. Immediately following exposure to the microwave irradiation, animals were decapitated and blood was collected in heparinized containers. Levels of cGMP in specific brain regions were sin ificantly elevated only in animals exposed to cold stress. The cGMP increase in cerebellum was approximately 3 fold with significant increases also noted in 9 other regions. Cyclic AMP (cAMP) levels throughout the 16 regions of brain examined showed no significant response to either of the stressors. In the pituitary, the level of cGMP was elevated in animals exposed to cold stress, while the cAMP level tended to increase only in the immobilized group. In the regions noted, the cGMP response to cold exposure was not affected by bilateral lesions of the locus coeruleus. Plasma prolactin levels were elevated over 10 fold in the immobilized group, while only rising 2 fold in the cold stress group. Levels of corticosterone were significantly increased while growth hormone levels tended to decrease in both the immobilized and cold stress groups. It would appear from our data that regional brain cGMP and plasma prolactin respond differentially to 2 stressors while plasma corticosterone and growth hormone respond less specifically. The specifics of the stressful stimuli may be crucial in determining the nature of the neurochemical and hormonal response to stress in the rat. It has been previously observed that the hormonal response to stress varies depending upon the nature of the specific stressful stimulus (41). Our data extend this observation in a different model and demonstrate specificity in a neurochemical response for the first time. Future work will include examination of sex differences in neurochemical and hormonal responses to stress, as well as neurochemical mechanisms involved in habituation to chronic exposure to stress.

Because many of our neurochemical studies require the use of highintensity microwave irradiation to accomplish enzyme inactivation, we have continued to initiate improvements in, and document limitations of, the microwave technology. Modifications of our present microwave power source, Varian PPS-2.5, introducing electronic circuitry to precisely control power delivered to the animal as well as exposure duration, have been completed by Peter Brown in the Department of Microwave Research and are presently in operation. Studies have continued in conjunction with Dr. Om Gandhi and Department of Microwave Research to develop an appropriate waveguide applicator to achieve more uniform enzyme inactivation in the brain. In collaboration with Dr. Gandhi we have produced and tested a waveguide applicator suitable for high energy exposures at 985 MHz. We have tested the uniformity of inactivation using the histochemical technique for succinic dehydrogenase activity (44). While the pattern of energy absorption at 2450 MHz is extremely sensitive to rotation of the load, the pattern of absorption at 985 MHz is remarkably independent of rotation. We are grateful to Dr. Larsen, Chief, Department of Microwave Research for the suggestion that we investigate the effects of simultaneous exposure to two frequencies. This innovation

is being explored collaboratively.

There is ample evidence that stress releases catecholamines from the adrenal medulla and by some indications, from brain neurons as well (6-12, 42). Although neuronal firing rates are one determinant of release (45), local presynaptic receptors are reported to influence releasability of brain NE (46). The latter work was done with exogenous, labelled NE. Such a model could prove misleading in that numerous storage compartments may be labelled, and therefore, specificity limited. Our laboratory has developed the first in-vitro model of release of endogenous NE from brain tissue (47). This advance permits us to examine the effects of acute and chronic stress or injury on pre-synaptic modulation of transmitter release. Such studies would not have been feasible without the development of our in-vitro model. We have previously measured the release of NE in vitro from hypothalamus, a region with a high NE concentration, in response to depolarizing concentrations of KCl, (47). We also reported that DA was released from striatum, a region rich in DA, after incubation with KCl (48). We have now extended our investigation to other regions of rat brain that have much lower catecholamine levels. We find detectable release of NE and DA in response to KCl in many regions of brain. The release of NE and DA in response to KCl stimulation was examined in 6 regions: cortex, hippocampus, hypothalamus, striatum, combined accumbens-olfactory tubercle, and substantia nigra. NE release in response to KCl was detectable in all regions except striatum. Amounts released after KCl (expressed as % control) were cortex (313%), hippocampus (227%), hypothalamus (225%), accumbenstubercle (278%), s.nigra (155%). KCl stimulated release of DA was detected in 3 regions: striatum (414%), accumbens-tubercle (282%), and hypothalamus (312%). DA was measurable in filtrates from the s.nigra but levels from control and KCl stimulated samples were equal. DA release was not detectable from cortex or hippocampus.

#### II. Neurochemical Sequelae to Penetrating Cerebral Injury

Post traumatic epilepsy (PTE) is a chronic, disabling complication of penetrating missile injuries of the brain. Such injuries are most commonly produced under conditions of warfare and produce PTE in more than 40% of soldiers so injured (49,50). PTE is much less frequent following closed head injuries typical of civilian accidents (49,51). High risk of PTE is associated with depth of injury, hypesthesia, coma, presence of non-viable cerebral tissue, parietal location of injury, occurrence of a torn dura and occurrence of infection (49,50,51). Despite advances in pharmacotherapy of seizures and significant reduction of incidence of infection since WW I, the incidence of PTE was only slightly reduced in the Korean War (52). The frequent observation of an "incubation period" of several months between the causative trauma and the first seizure suggests an opportunity to develop prophylactic treatment to prevent PTE and chronic disability.

Studies directed at the PTE problem have been initiated in our laboratory. A collaborative study with Dr. Barry Hoffer at NIMH has yielded preliminary data suggestive of a correlation between induced seizures in hippocampal tissue and elevated levels of cGMP. Additional studies on the neurochemical sequelae of cerebral injury examined the hyperdipsiaurinary destruction syndrome observed following dysfunction of the locus coeruleus (LC) (53). Although our lesions produced histological evidence of destruction of the LC and greater than 80% depletion of cortical NE, only 25% of subjects developed hyperdipsia and urinary dysfunction. This suggests that the syndrome is not caused by NE insufficiency and may be caused by destruction of a brain area near the LC rather than destruction of the LC itself.

Project 3M161102BS01 RESEARCH ON MILITARY DISEASE

Work Unit 128 Mechanism of Response to Military Stress

#### Literature Cited.

#### References:

- 1. Henkin, R.I., and Knigge, K. Effect of sound on the hypothalamic-pituitary-adrenal axis. Am. J. Physiol. 204:710, 1963.
- 2. Dunn, J., Scheving, L., and Millet, P. Circadian variation in stress-evoked increases in plasma corticosterone. Am. J. Physiol. 222(2):402-406, 1972.
- 3. Kokka, G.J.F., George, R., and Elliott, H.W. Growth hormone and ACTH secretion: evidence for an inverse relationship in rats. Endocrinology 90(3):735-743, 1972.
- 4. Takahashi, K., Daughaday, W.M., and Kipnis, D.M. Regulation of immunoreactive growth hormone secretion in male rats. Endocrinology 88:909-917, 1971.
- 5. Brown, G.M., Martin, J.B. Corticosterone, prolactin, and growth hormone responses to handling and new environment in the rat. Psychosom. Med. 36(3):241-247, 1974.
- 6. Corrodi, M., Fuxe, K., and Hokfelt, T. The effect of immobilization stress on the activity of central monoamine neurons. Life Sci. 7:107-112, 1968.
- 7. Korf, J., Aghajanian, G.J., and Roth, R.M. Increased turnover of norepinephrine in the rat cerebral cortex during stress: role of the locus coeruleus. Neuropharmacology 12:933-938, 1971.
- 8. Thierry, A.M., Blanc, G., and Glowinski, J. Effect of stress on the disposition of catecholamines localized in various intraneuronal storage forms in the brain stem of the rat. J. Neurochem. 18:449-461, 1971.
- 9. Thierry, A.M., Javoy, F., Glowinski, J., and Kety, S.S. Effects of stress on the metabolism of norepinephrine, dopamine and serotonin in the central nervous system of the rat. I. Modifications of norepinephrine turnover. J. Pharm. Exp. Ther. 163:163-171, 1968.
- 10. Bliss, E. Relationship of stress to brain serotonin and 5-hydroxyindoleacetic acid. J. Psychiat. Res. 9:71-80, 1972.
- 11. Morgan, W. Effect of immobilization stress on serotonin content and turnover in regions of the rat brain. Life Sci.  $\underline{17}$ :143-150, 1975.

- 12. Bliss, E. Relationship of stress and activity to brain dopamine and homovanillic acid. Life Sci. 10:1161-1169, 1971.
- 13. Mao, C.C., Guidotti, A., and Costa, E. Interations between  $\gamma$ -aminobutyric acid and guanosine cyclic 3',5'-monophosphate in rat cerebellum. Molec. Pharm. 10:735-745, 1974.
- 14. Dinnendahl, V. Effects of stress on mouse brain cyclic nucleotide levels <u>in vivo</u>. Brain Res. <u>100</u>:716-719, 1975.
- 15. Holmes, H., Rodknight, R., and Kapoor, R. Effects of electroshock and drugs administered in vivo on protein kinase activity in rat brain. Pharmacol. Biochem, and Behav. 6:415-419, 1977.
- 16. Huang, M.J. Accumulation of cyclic adenosine monophosphate in incubated slices of brain tissue. I. Structure-activity relationships of agonists and antagonists of biogenic amines and of tricyclic tranquilizers and antidepressants. Medicinal Chem. <u>15</u>:458, 1972.
- 17. Forn, J. Adenosine 3',5'-monophosphate content in rat caudate nucleus: demonstration of dopaminergic and adrenergic receptors. Science 186:1118-1120, 1974.
- 18. Burkard, W.P. Catecholamine induced increase of cyclic adenosine 3',5'-monophosphate in rat brain in vivo. J. Neurochem. 19:2615-2619, 1972.
- 19. Leonard, B.E. The effect of two synthetic ACTH analogues on the metabolism of biogenic amines in the rat brain. Arch. Int. de Pharmacodynamie 207:242-253, 1974.
- 20. Mao, C.C. The regulation of cyclic guanosine monophosphate in rat cerebellum: possible involvement of putative amino acid neurotransmitters. Brain Res. 79:510-514, 1974.
- 21. Steiner, A.L., Kipnis, D.M., Utiger, R., and Parker, C.W. Radioimmunoassay for the measurement of adenosine 3',5'-cyclic phosphate. Proc. Nat. Acad. Sci. 64:367, 1969.
- 22. Steiner, A.L., Wehmann, R.E., Parker, C.W., and Kipnis, D.M. Radioimmunoassay for the measurement of cyclic nucleotides. In Advances in Cyclic Nucleotide Research, Vol. 12, Ed. by P. Greengard, G. Alan Robison, and R. Paoletti. Raven Press, New York, 1972.
- 23. Graham, L.T., and Aprison, M.L. Fluorometric determination of aspartate, glutamate, and  $\gamma$ -aminobutyrate in nerve tissue using enzymatic methods. Analytical Biochem. 15:487-497, 1966.
- 24. Ferrendelli, D.A., Steiner, A.L., McDougal, D.B. Jr., and Kipnis, D.M. The effect of oxotremorine and atropine on cGMP and cAMP levels in mouse cerebral cortex and cerebellum. Biochem. and Biophys. Rsch. Comm. 41(4):1061-1067, 1970.

- 25. Huang, M.J. Accumulation of cyclic adenosine monophosphate in incubated slices of brain tissue. I. Structure-activity relationships of agonists and antagonists of biogenic amines and of tricyclic tranquilizers and antidepressants. Medicinal Chem. 15:458, 1972.
- 26. Brown, J.H. Stimulation by dopamine of adenylate cyclase in retinal homogenates and of adenosine-3',5'-cyclic monophosphate formation in intact retina. Proc. Nat. Acad. Sci. 69(3):539-543, 1971.
- 27. Kebabian, J.W. Petzold, G.L., and Greengard, P. Dopamine-sensitive adenylate cyclase in caudate nucleus of rat brain, and its similarity to the "dopamine receptor". Proc. Nat. Acad. Sci. 69:2145-2149, 1972.
- 28. Lenox, R.H., Meyerhoff, J.L., and Wray, H.L. Regional distribution of cyclic nucleotides in rat brain as determined after microwave fixation technique. Proc. Soc. for Neurosciences Fourth Annual Meeting, 1974.
- 29. Balcom, G.J., Lenox, R.H., and Meyerhoff, J.L. Regional  $\gamma$ -aminobutyric acid levels in rat brain determined after microwave fixation. J. Neurochem. 24:609-613, 1975.
- 30. McEwen, B. Corticosterone binding to hippocampus: immediate and delayed influences of the absence of adrenal secretion. Brain Res. 70:321-334, 1974.
- 31. Nakagawa, K., and Kuriyama, K. A modulating role of pituitary-adrenal axis in cerebral metabolism of adenosine 3',5'-monophosphate. J. Neurochem. 27:609-612, 1975.
- 32. Azmitia, E.C. Corticosterone regulation of tryptophan hydroxylase in midbrain of the rat. Science 166:1274-1276, 1969.
- 33. Lorens, S. Regional 5-hydroxytriptamine following selective midbrain raphe lesions in the rat. Brain Res. 78:45-46, 1974.
- 34. Dahlstrom, A., and Fuxe, K. Evidence for the existence of monoamine-containing neurons in the central nervous system. I. Demonstration of monoamines in the cell bodies of brainstem neurons. Acta Physiol. Scand. 62, Suppl. 232:1-55, 1964.
- 35. Mosko, S.S., and Jacobs, B.L. Midbrain raphe neurons: sensitivity to glucocorticoids and ACTH in the rat. Brain Res. 89:368-375, 1975.
- 36. Foote, W.E. Effect of hydrocortisone on single unit activity in midbrain raphe. Brain Res. 41:242-244, 1972.

- 37. Martin, S.M., Moberg, G.P., and Horowitz, J.M. Glucocorticoids and the hippocampal theta rhythm in loosely restrained, unanesthetized rabbits. Brain Res. 93:535-542, 1975.
- 38. Aghajanian, G.K. Serotonin: elease in the forebrain by stimulation of midbrain raphe. Science 156:402, 1967.
- 39. Sheard, M.H., and Aghajanian, G.K. Stimulation of midbrain raphe neurons: ehavioral effects of serotonin release. Life Sci. 7:19-25, 1968.
- 40. Brostram, M.A. Adenosine 3',5'-Monophosphate in glial tumor cells treated with glucocorticoids. Molec. Pharm. 10:711-720, 1974.
- 41. Mason, J.W. A re-evaluation of the concept of "non-specificity" in stress theory. J. Psychiat. Res. 8:323-333, 1971.
- 42. Keim, K., and Sigg, E.B. Plasma corticosterone and brain catecholamines in stress: effect of psychotropic drugs. Pharmacol. Biochem. and Behav. 4:289-297, 1976.
- 43. Lenox, R.H., Gandhi, O.P., Meyerhoff. J.L., and Grove, H.M. A microwave applicator for <u>in vivo</u> rapid inactivation of enzymes in the central nervous system. IEEE Trans. Microwave Theory and Tech. 58-61, Jan 1976.
- 44. Nachlas, M.M., Tsou, K.-C., De Souza, E., Cheng, C.-S., and Seligman, A.M. Cytochemical demonstration of succinic dehydrogenase by the use of a new p-nitrophenyl substituted ditetrazole. J. Histochem. Cytochem. 5:420-436, 1957.
- 45. Korf, J., Aghajanian, G.K., and Roth, R.H. Stimulation and destruction of the locus coeruleus: opposite effects on 3-methoxy-4-hydroxyphenylglycol sulfate levels in the rat cerebral cortex. Eur. J. Pharmacol. 21:305-310, 1973.
- 46. Starke, K., and Montel, H. Involvement of α-receptors in clonidine-induced inhibition of transmitter release from central monoamine neurones. Neuropharmacology 12:1073-1080, 1973.
- 47. Kant, G.J., and Meyerhoff, J.L. Release of endogenous norepinephrine from rat hypothalamus in vitro. Life Sci. 20:149-154, 1977.
- 48. Balcom, G.J., and Meyerhoff, J.L. Release of endogenous norepinephrine and dopamine in vitro. Proc. Soc. for Neuroscience, Sixth Annual Meeting, 1976.
- 49. Caviness, V.S. Epilepsy: A late effect of head injury. In: The late effects of head injury. pp. 193-201, Ed. by A.E. Walker, W.F. Caviness, and M. Critchley. Charles C. Thomas.

- 50. Russell, W.R. The development of grand mal after missile wounds of the brain. Johns Hopkins Med. J. 122:250, 1968.
- 51. Jennett, B., Miller, J.D., and Braakman, R. Epilepsy after non-missile depressed skull fracture. J. Neurosurg. 41:208-216, 1974.
- 52. Caviness, W.T., Walker, A.E., and Ascroft, P.B. Incidence of post-traumatic epilepsy in Korean veterans as compared with those from World War I and World War II. J. Neurosurg. 19:122-129, 1962.
- 53. Osumi, Y., Oishi, R., Fujiwara, H., and Takaori, S. Hyperdipsia induced by bilateral destruction of the locus coeruleus in rats. Brain Res. 86:419-427, 1975.

#### Publications:

- 1. Kant, G.J., and Meyerhoff, J.L. Release of endogenous norepinephrine from rat hypothalamus in vitro. Life Sci. 20:149-154, 1977.
- 2. Koob, G.F., Sessions, G.R., Kant, G.J., and Meyerhoff, J.L. Dissociation of hyperdipsia from the destruction of the locus coeruleus in rats. Brain Res. <u>116</u>:339-345, 1976.
- 3. Sessions, G.R., Kant, G.J., and Koob, G.F. Locus coeruleus lesions and learning in the rat. Physiol. and Behav. 17:853-859, 1976.
- 4. Iwamoto, E.T., Craves, F.B., Loh, H.H., Meyerhoff, J.L., and Way, E.L. Axonal transport in nigro-neostriatal neurons during morphine tolerance development and abstinence in rats. J. Neurochem. 28:285-292, 1977.
- 5. Balcom, G.J., and Meyerhoff, J.L. Release of endogenous norepinephrine and dopamine <u>in vitro</u>. Proc. Soc. for Neuroscience, Sixth Annual Meeting, 1976.
- 6. Lenox, R.H., Balcom, G.J., and Meyerhoff, J.L. Regional levels of norepinephrine and dopamine in rat brain after microwave fixation at 2450 or 985 MHz: possible diffusion artifact. Proc. Soc. for Neuroscience, Sixth Annual Meeting, 1976.
- 7. Sessions, G.R., Salwitz, J.C., and Kant, G.J. Behavioral effects of lesions of the locus coeruleus. Proc. Soc. for Neuroscience, Sixth Annual Meeting, 1976.
- 8. Meyerhoff, J.L., Lenox, R.H., and Brown, N.D. Amino acid levels in mouse brain determined after microwave fixation. Proc. Soc. for Neuroscience, Sixth Annual Meeting, 1976.

DESEADON	AND TECHNOLOGY	WOOK HINT	CIIMMADY	1. AGEN	CY ACCESSIONS	2. DATE OF SA	IMMARY <sup>6</sup>		CONTROL SYMBOL
			S. WORK SECURITY	DA C	A 6440	77 10		1	RAE(AR)636
76 10 01	D.Change	U.	U. WORK SECURITY	NA NA			ON SPECIFIC	ACCESS	A WORK UNIT
10 NO./CODES:*	PROGRAM ELEMENT		T NUMBER		REA NUMBER	ь		IT NUMBER	
& PRIMARY	61102A	3M161102F		1	00		129		
b. CONTRIBUTING	OTTOZA	SWILLIAM	SUL		-				
c. CONTRIBUTING	CARDS 114F								
	Security Classification Code								
(U) Parasit	ic Diseases	of Militar	y Importanc	e					
002600 Biol									
13. START DATE	rogy	14. ESTIMATED CO	MPLETION DATE	IS FUND	HIG AGENCY		16. PERFOR	MANCE MET	HOD
54 09		CONT		DA	1	1	C. In	-House	
17. CONTRACT/GRANT				-	OURCES ESTIMAT	A PROFES	SIONAL MAN Y		DS (In thousands)
A DATES/EFFECTIVE:	NA	EXPIRATION:			PRECEDING				
NUMBER:				FISCAL	77 CURRENT	1	5		418
C TYPE:		4 AMOUNT:		TEAR				1	415
KIND OF AWARD:	ORGANIZATION	f. CUM. AMT	·	20. PER	78		3		41)
				4			my Inct	itute	of Research
Walter	Reed Army In	stitute of	Research		Div of		цу 11150.	Tuuce	or nesear
ADDRESS.* Washington, D. C. 20012				ADDRESS:					
				Washington, D. C. 20012  PRINCIPAL INVESTIGATOR (Pumish SEAN II U.S. Academic Incilintary)					
				1				ile [netifution	,
RESPONSIBLE INDIVIDUAL  CARRESON RAPMIND, COL.					BURKE,				
NAME: GARRISON RAPMUND, COL VELEPHONE: (202 576-3551				The second	HONE: (20	2) 576-2	2273		
TELEPHONE: (LUC )/(U-J)//L					TE INVESTIGATO				
				HAME:	MOON.	A. P.	DA		
	elligence no		ed	NAME:	,				
	EACH with Society Classifi	-	U) Parasite	; (U)	Schisto	somiasis	s; (U) 1	Pathol	ogy;
(U) Primate	(U) Chemoti	nerapy; (U	) Immunolog	y; (U	) Trypan	osomoas	is: (U)	Filar	iasis
	e purpose of cal aspects								
gaining a h	etter underst	tanding of	natural su	scent	ihility	aconire	ed resis	stance	and ef-
	of therapeu								
these infec	-				,,			· cu un c	
24 (U) Thro	ough careful p	perusal of	pertinent	liter	ature an	d discus	ssion wi	ith oth	ner
	both classic								
	10-77 09 An								
	ras used in th								
	for the EATR								
	hieved using								
	ous system an								
	of mice as lo								
	luorescent ar								
	Walter Reed A								
30 Sep 77.									
			292						

Project 3M161102BS01 BASIC RESEARCH ON MILITARY DISEASES

Work Unit 129 Parasitic Diseases of Military Importance

Investigators.

Principal: COL J.C. Burke, MSC

Associate: W.L. Bowie, D.E. Davidson, C.L. Diggs, J.J. DiConza,

J.A. Gold, R.W. Gore, W.T. Hockmeyer, R.F. Jackson, F. von Lichtenberg, R. McDermott, C.A. Montgomery, G.F. Otto, R.H. Perry, S.M. Phillips, B.C. Redington, W.A. Reid, R. Roscinski, M. Schoenbechler, J.M. Shields,

J.R. Staub, M.S. Williams

1. Neutralization inhibitions using glycoprotein extract of surface membranes of Trypanosoma rhodesiense.

Trypanosomes persist in the blood of infected animals even though neutralization reactions show that there are protective antibodies in the serum. According to Cross, structural changes in the glycoproteins of the parasite's cell membrane produce antigenically different cells which are not destroyed by the host. Gore, et al showed that glycoprotein extracts of cell membranes could induce a strain and variant immunity when injected into mice.

This study investigated the strain and variant specificity of extracted glycoproteins of cell membranes of <u>Trypanosoma</u> <u>rhodesiense</u> (EATRO 1886) by inhibiting the ability of immune sera to protect mice.

Sera for this study were obtained from chimpanzees infected with <u>T. rhodesiense</u> EATRO 1886 and Wellcome strains and were stored at -20% until used. Albino ICR mice (Walter Reed strain) were used to test neutralization reactions.

Glycoproteins were obtained as follows: Trypanosomes were extracted for 16-20 hours at 40 in 3M KCl. This parasite extraction mixture was centrifuged for 2 hours, the supernatant was recovered and diluted 15 volumes with distilled water and concentrated by negative pressure dialysis. After concentration it was separated by column chromatography using G-200 Sephadex and subsequent sucrose gradient electrophoresis.

Neutralization reactions were performed essentially as described by Soltys and modified for inhibition studies as described below. Varying dilutions of serum from infected chimpanzees were made in Medium 199 containing 2% fetal calf serum (serially diluted 1:10-1:80 and 0.3 ml were placed in test tubes. Into each tube one milliliter of viable trypanosomes were added containing  $10^3/\text{ml}$ . The tubes were mixed well by shaking and allowed to stand at room temperature for 30 min., after which 0.25 ml of the serum-parasite mixture was injected intraperitoneally into mice. The mice were observed for 21 days for survivors. The control mice using sera of noninfected chimpanzees died of trypanosomiasis by day seven.

Neutralization inhibition studies were done as above except that prior to exposing viable trypanosomes to the diluted sera, it was absorbed by a given amount of glycoprotein extract for a period of two hours at 37°C.

The neutralization inhibition reactions were optimized by absorbing the sera under the following conditions: 1) varying the amount of glycoprotein extract, 2) varying the time and temperature, and 3) varying the serum dilution. It was determined that 0.3 ml of a serum dilution of 1:20 absorbed with 3.28ug of glycoprotein extract at 37°C for two hours was optimal. All further tests were done under these conditions.

TABLE I

# EFFECTS OF TIME AND TEMPERATURE ON THE EFFICIENCY OF SPECIFIC ANTIBODY ABSORPTION AS SHOWN BY MOUSE SURVIVAL IN THE TRYPANOSOMAL NEUTRALIZATION TEST

		Time of A	Absorptio	n (Hou	(2)	
	24	4	2	1	0.5	controls
ပ္ပု37	0*	2	0	1	1	5
<u>e</u> 22	0	1	0	2	3	5
₽ 4	0	0	0	2	4	5

The soluble antigen fluorescent antibody (SAFA) test was performed as described by Sadun and Gore using the Turner fluorometer to give objective end point titer determinations.

The effect of time and temperature on the efficiency of specific protective antibody absorption of serum from infected chimpanzees is shown in Table I, by the mouse survival rate in the trypanosomal neutralization reaction. Two hours of absorption at 37° was selected as optimal for our study although other times and/or temperatures could have been used.

To determine the optimal amount of glycoprotein extract to use in this study, a volume of 0.3 ml of a 1:20 serum dilution was absorbed with varying amounts of the glycoprotein extract. It can be readily seen in Table II that 3.28  $\mu g/0.3$  ml of 1:20 dilution of serum was effective in reducing the survivors to zero. This shows that 21.0  $\mu g$  of glycoprotein extract could remove all of the protective antibody in 1 ml of immune serum.

Strain specificity was demonstrated using serum collected early and late during the course of the infection from two chimpanzees. Table III shows the effect of diluting the serum on survival in the neutralization test. However, it is abundantly clear that homologous glycoprotein extract mediates the protection as shown by neutralization inhibition as opposed to no inhibition by the heterologous glycoprotein extract.

TABLE II

THE EFFECT OF ABSORBING SERUM FROM A CHIMPANZEE INFECTED WITH T. RHODESIENSE WITH VARYING AMOUNTS OF GLYCOPROTEIN ANTIGENS ON THE NEUTRALIZATION OF TRYPANOSOMES

$\mu$ g of Antigen/0.3	ml Dil Serum	Number of Mice Surviving (Groups of 5)		
Normal Serum				
	0.0	0		
	1.64	0		
Immune Serum	4.1	0		
	3.28	0		
	2.46	1		
	1.64	1		
	0.82	5		
	0.42	5		
	0.0	5		

Sera collected from four chimpanzees at definite intervals were tested and the results compared for variant specificity. In Table IV the inhibition results for each chimpanzee's sera differs in survival rates at different times during the infection. However, the protection was again removed after 20-32 weeks of the infection in all animals tested. Sera which had retained their protective ability after absorption under standard conditions were exhaustively absorbed with increasing amounts of glycoprotein. The ability to protect mice was retained after 21 days.

TABLE III

HOMOLOGOUS AND HETEROLOGOUS GLYCOPROTEIN ANTIGEN ABSORPTION OF SERA FROM CHIMPANZEES INFECTED WITH TRYPANOSOMA RHODESIENSE.

	Serum col	Serum collected early in the infection				Serum collected late in the infection	infection
		Numbers of mic	ce surviving in t	Numbers of mice surviving in trypanosomal neutralization	utralization		
Source of	Non-absorbed	Glycoprotein Absorbed	n Absorbed	Serum	Non-	Glycoprotein Absorbed	n Absorbed
Serum	000000000000000000000000000000000000000	KCI-X 1886	KCI-X Well	Dilution	Dag losge-lion	KCI-X 1886	KCI-X Well
	<b>å</b>	2	2	1:10	D.	0	2
Chimpanzee	2	2	S.	1:20	S	0	2
T. rhodesiense	2	2	S	1:40	2	0	2
154110 1000	2	4	2	1:80	2	0	ß
	-	2	S.	1:100	2	0	ß
Non-Infected Chimpanzee	0	0	0	1:10	0	0	0
	S	വ	-	1:10	2	2	0
Chimpanzee	4	2	-	1:20	2	4	0
T. rhodesiense	-	-	0	1:40	0	0	0
(Melicollie)	0	0	0	1:80	0	0	0
	0	0	0	1:100	0	0	0

TABLE IV

COMPARISON OF RESULTS OF NEUTRALIZATION AND NEUTRALIZATION INHIBITION USING SERUM FROM CHIMPANZEES INFECTED WITH

T. RHODESIENSE STRAIN (EATRO 1886)

			С	himpanze	e Numbe	ers		
Week of Infection	P1	09	4	78	Me	881	M	759
	Neut.	Inhib.	Neut.	Inhib.	Neut.	Inhib.	Neut.	Inhib
0	0*	0	0	0	0	0	0	0
2	0	0	0	0	5	0	5	0
4	5	5	5	0	5	3	5	0
6	5	5	5	0	4	1	5	0
8	5	5	4	5	5	4	5	2
10	5	5	5	5	5	1	5	4
12	5	5	5	1	5	1	5	1
14	5	5	5	5	4	0	5	0
20	5	5	5	5	5	0	5	0
28	5	3	5	4	5	0	5	0
32	5	0	5	0	5	0	5	0
66	5	0	5	0	5	0	5	0

<sup>\*</sup>Numbers of Mice Surviving (Groups of 5)

The antigen variants can be easily demonstrated by the differing antibody populations produced by the mammalian hosts as shown by Soltys, Russell and Leupold. Although the biological mechanism is not clearly defined, it was postulated early that the antigens expressed were located in the cell membrane, and different populations of trypanosomes could be detected by serological test. Evidence confirming this was presented by Fruit, et al and Viekerman and Luckens when labeled anti-variant antibody reacted with the surface membrane of bloodstream trypanosomes. Recently Cross followed by Gore, et al extracted and purified a glycoprotein from the surface coat of blood from trypanosomes which reacts in serological tests and induces an immunity specific for the same parasites.

Using the membrane glycoprotein as extracted by Gore, et al from  $\underline{\mathbf{T}}$ .  $\underline{\mathbf{r}}$ hodesiense strains EATRO 1886 and Wellcome, the protection given by sera from chimpanzees infected with these strains could be completely removed. This was effectively done by absorbing the sera from infected chimpanzees with the glycoprotein extract and using the sera in the trypanosoma neutralization test of Soltys.

Evidence supporting strain specificity was obtained when the result of neutralization study on sera from chimpanzees infected with Wellcome strain was absorbed with EATRO 1886 strain and vice versa, namely all of the mice in the cross absorption study survived suggesting that a glycoprotein of heterologous origin would not react with and remove the protective antibody. However, when absorbed with the homologous antigen protective antibody was removed, therefore, the mice died of trypanosomiasis.

Variant specificity of the extracted glycoprotein was demonstrated in the serially collected sera of four chimpanzees infected with  $\underline{\mathbf{T}}$ . rhodesiense strain EATRO 1886. In the sera which retained its protective antibody exhaustive absorption with glycoprotein of EATRO 1886 did not remove the protection from the sera.

The difficulty of producing an immunogen capable of invoking a protective immunity is becoming painfully clear. There are antigenic differences in strains of T. rhodesiense, and these strains show differences in each variant parasite populations with changes in amino acid sequences and carbohydrate structures of the glycoprotein molecule in the surface of the parasite membrane. The number of possible variants due to glycoprotein structural differences are exponentrally staggering. However, it is surprising and evealing to learn that the ability of the homologous glycoprotein of T. rhodesiense EATRO 1886 stabalide to again remove the protection in the serum obtained late in the course of the infection. This suggests that these exist as basic antigenic type coded into each strain of parasites. This basic antigenic type may be geographically circumscribed. This may permit the production of a vaccine for the indigents of that area. Further it can be postulated that different strains may be antigenically equivalent at some point of variant population regardless of glycoprotein structural complexities.

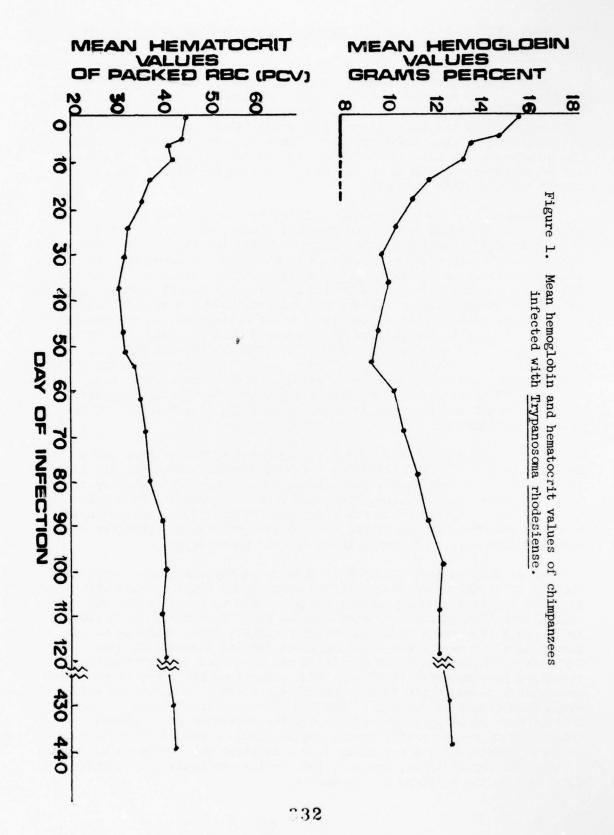
### 2. Immunological basis for the anemia of sub-human primates infected with Trypanosoma rhodesiense.

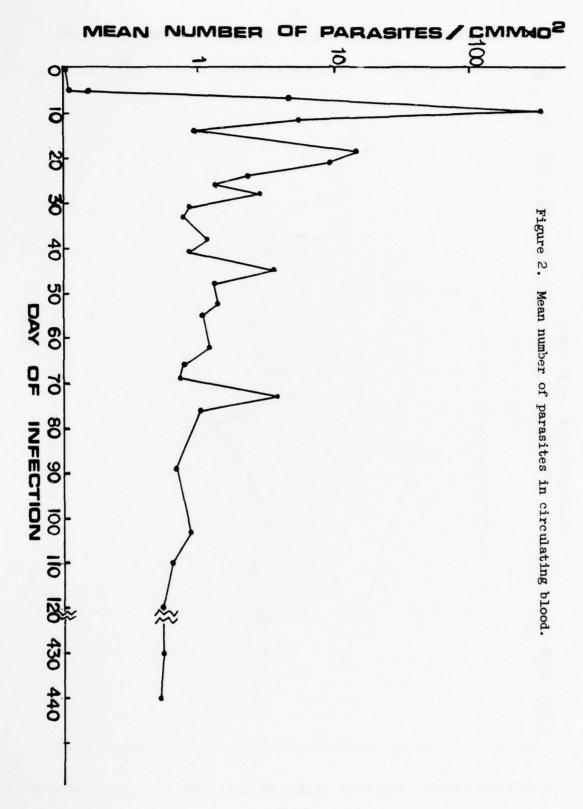
Four chimpanzees inoculated intravenously with 2000 <u>Trypanosoma</u> rhodesiense trypomastigotes (EATRO 1886) developed an anemia as early as 7 days after infection (Figure 1). Parasitemia had reached a peak (>30,000/cmm) by day ten (Figure 2) at which time specific antibodies were detected by the soluble antigen fluorescent antibody test (Figure 3). The hematocrit and hemoglobin (Figure 1) had dropped as much as 11% and 15% of preinfection values by day 10 and as much as 30% and 25% respectively 19 days after infection.

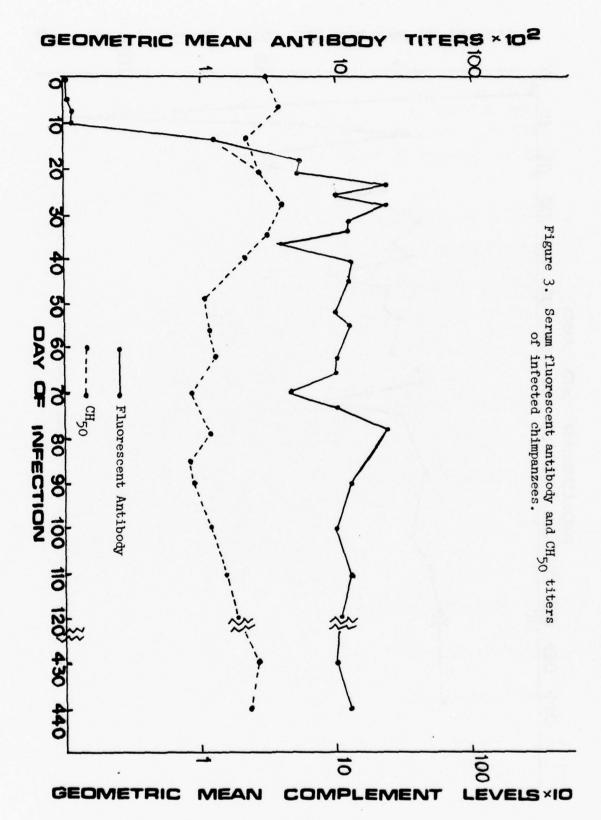
Agglutination of erythrocytes (RBC's) from uninfected or infected chimpanzees by peanut lectin was studied; whereas RBC's of uninfected chimpanzees did not agglutinate, those from all infected chimpanzees were agglutinated early in the infection. Furthermore, RBC's exposed to sonicates of trypanosomes in vitro were also agglutinated by the lectin. All of these reactions were inhibited by lactose. Others have shown that antibodies (T-agglutinins) to the RBC receptor involved in this reaction exist in the sera of most vertebrates; these antibodies can be detected by their ability to agglutinate RBC's treated with neuraminidase (N'ase). Accordingly, we studied the agglutination of N'ase treated RBC's by chimpanzee serum collected during the course of the infection (Figure 4). Agglutinating antibody titers were present prior to infection; rose rapidly to a peak at about day 20, and remained elevated thereafter. Positive indirect Coombs activity was detected on days 10, 14, 20 and 40 of the infection (Figure 5).

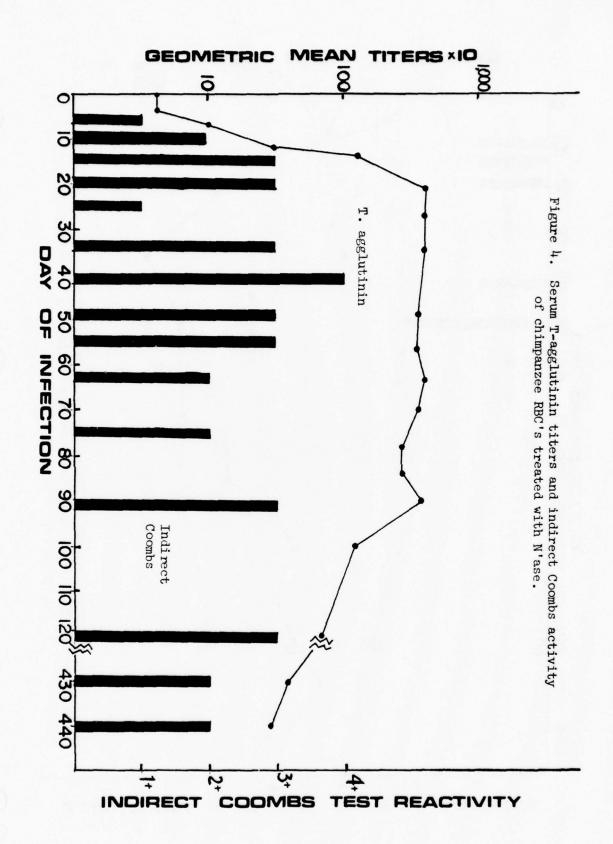
Complement depletion occurred during the infection; there was an average 30% drop in CH50 titers by day 50 of the infection (Figure 5); two animals were transiently completely acomplementemic. Antibody titers to C3/44 (immunoconglutinins) increased from the preinfection level of 1:100 to 1:6400 by day 35 of the infection (Figure 5). Liver and spleen biopsies revealed binding of RBC's to hepatic macrophages (Kupffer cells) and extensive erythrophagocytosis by splenic macrophages.

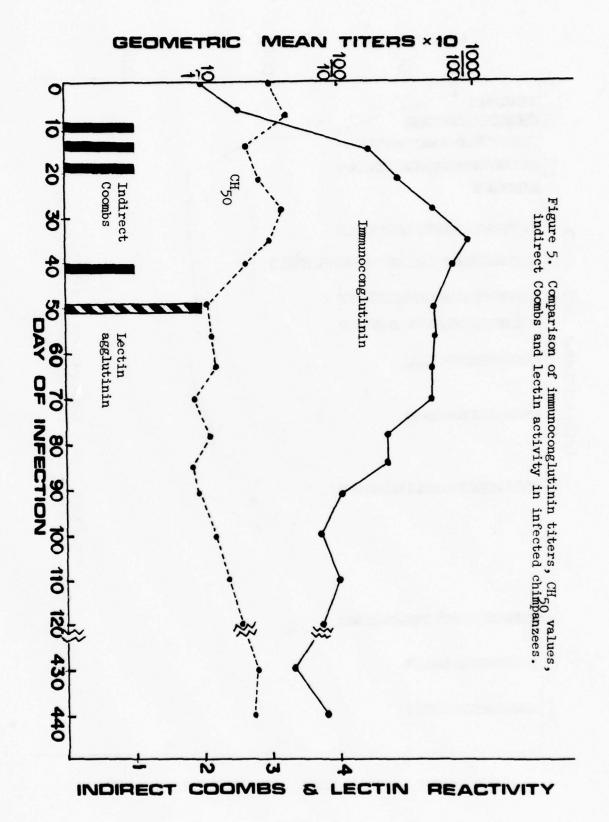
These data suggest a number of intriguing explanations. Agglutinability of erythrocytes from infected animals and RBC's treated with N'ase in vitro suggests that trypanosomes may be able to expose a RBC receptor during infection in a manner analogous to N'ase treatment. Such altered RBC's would then react to the naturally occurring serum antibody to the peanut lectin receptor and fix complement forming immune complexes. Trypanosome antigen-antibody complexes may also bind to erythrocytes resulting in complement fixation. Such sensitized RBC's may be much more susceptible to adherence to macrophages via Fc and C3b receptors which would then result in removal of the RBC from the circulation. Further, this process could be modulated through the depletion of complement components by reaction with immunoconglutinins. A number of variations on these hypotheses are obviously also consistent with these data. Most of these interpretations, however, involve the implication of immune complexes in the etiology of the anemia.











3. Blastogenic responses of spleen cells from mice infected with Trypanosoma rhodesiense uninfected mice, and immunized mice to mitogens and a glycoprotein antigen.

When spleen cells from mice infected with some bacteria or helminths are cultured along with phytohemagglutinin (PHA) or lipopolysaccharides (LPS) or specific antigens they undergo blastogenesis regularly. We were unable to induce blastogenesis in spleen cells from mice infected with Trypanosoma rhodesiense using antigen from sonicated parasites or a glycoprotein extract of parasite cell membranes. However, using PHA or LPS there was blast transformation the first three or four days after infection which disappeared by the 4th or 5th day of the infection. With cells of uninfected animals there was no blast transformation using the antigens, but there was a smaller amount with both PHA and LPS. Therefore, the blastogenic responses of spleen cells from uninfected mice, immunized mice, and infected mice were compared.

Spleens of uninfected mice were gently teased and passed through a 50 mesh stainless steel wire screen. These cells were washed once in 1640 RPMI medium and shocked with phosphate buffered 0.83% ammonium chloride solution to lyse contaminating red blood cells. The lymphoid cells remaining were washed three times in medium as described above. They were then cultivated in 1640 RPMI medium containing 5% fetal calf serum (106/ml) with PHA (Difco) diluted 1:20 through 1:100, and LPS (Escherichia coli), 5  $\mu$ g to 50  $\mu$ g per culture. After 48 to 120 hours they were pulsed with 3H thymidine during the last 25 hours of growth to determine blastogenesis. The optimal condition for maximum 3H thymidine uptake was found to be 48 hours with a 1:60 dilution of PHA, and 72 hours with 50  $\mu$ g of LPS. The ratio of response was obtained by dividing the mean of 3 replicate culture counts per minute (CPM) of treated cells by the means of 3 replicate cultures CPM of untreated cells. The mean ratio of blastogenic response of cells from non-infected spleens was 4.2 for PHA and 4.7 for LSP treated cells.

Spleen cells from mice immunized with a total of  $12~\mu g$  of glycoprotein antigen were cultured under optimal conditions as described with and without glycoprotein antigen. Cells from infected mice were obtained at three to five day intervals for 24 days after infection and cultured as described.

The ratio of response of cells from uninfected mice treated with PHA was as much as 8 fold above untreated cells while the ratio with LPS treated cells was 7 fold more than with that of untreated cells. Increasing quantitites of antigen did not affect the blastogenic response of the PHA but there was as much as a 23% reduction in the non-infected cell response to LPS. There was no appreciable blastogenic response to glycoprotein antigen.

Spleen cells of mice immunized with glycoprotein antigen responded to PHA and LPS as well as to the glycoprotein antigen. However, with increasing quantities of glycoprotein the PHA response was reduced by as much as 20%.

Three days after infection spleen cells had ratios as high as 2.8 for PHA and 4.4 for LPS stimulation and very little or no response to glycoprotein antigen. However, as in the immunized animals, the cells showed diminishing responses by as much as 11% for PHA and 32% for LPS stimulation as the amount of antigen increased. By the fifth day after infection and until the end of the experiment blastogenic responses were never greater than those of the control cells cultured without treatment or stimulation.

These data suggest that the glycoprotein extract used as antigen in this system cannot trigger the blastogenic mechanism and initiate DNA synthesis. Apparently the glycoprotein antigen occupies or shares receptor sites on spleen cell membranes which inhibits or blocks the triggering of blastogenesis by the two mitogens, PHA and LPS.

### 4. The transfer of protection against T. rhodesiense by specific mouse spleen cell populations.

The purpose of the study was to investigate some aspects of immunity transferred with unfractionated immune mouse spleen cells from hyperimmune donors, and with specifically enriched spleen cell populations obtained by adherence to plastic and by fractionation in Sephadex G-290-anti-mouse gamma globulin columns.

Inbred C57BI6/J mice were hyperimmunized by a regimen of 5 weekly injections of 1 X 10<sup>7</sup> gamma irradiated <u>T. rhodesiense</u> (Wellcome strain) per mouse. Immune spleen cells were taken from these mice within 7 days after the last injection. For some experiments immune spleen cells were taken from these mice within 7 days after the last injection. For some experiments immune spleen cells were fractionated by adherence to plastic and characterized by particle latex phagocytosis and by esterase staining. Non-plastic-adherent fractions were treated by iron carbonyl and density centrifugation before fractionation into B- and T-enriched cell populations by immunoabsorbent column chromatography. Cells were characterized by the presence of surface immunoglobulin and E-rosette formation with sheep red blood cells.

Mice receiving 5 inoculations of 1 X 10<sup>7</sup> irradiated trypanosomes were immune to challenge infections of 200 trypanosomes. Spleen cells from these hyperimmune mice were used for the following studies.

The first experiments were designed to determine if immune spleen cells could convey protection against a challenge infection in recipient mice and how many spleen cells are needed to protect. At least 25 X 100 immune spleen cells were needed to protect. Normal spleen cells did not protect. Mice in these first experiments were challenged with trypanosomes 7 days after receiving cells. In the series of experiments the time course of protection transferred by immune spleen cells was determined. More specifically the questions of how soon after receiving immune cells can protection be confirmed and how long after receiving immune cells does the protection last. Mice that were infected 1 or 2 days before receiving

70 X 100 immune spleen cells died when challenged with trypanosomes. Mice given immune spleen cells on the same day as receiving the trypanosome infection only lived 2 days longer than the control mice receiving normal spleen cells. However, when immune spleen cells were given 1 day prior to infecting the mice were protected from the trypanosome challenge. This protection is viable for 21 days after receiving the immune spleen cells.

The first two series of experiments showed that immune spleen cells, from hyperimmune mice that had received 5 injections of irradiated trypanosomes, protected recipient mice from a homologous trypanosome challenge. The next group of experiments were designed to describe specific characteristics of the spleen cell responsible for transferring the protection. Spleen cells were separated into a macrophage enriched cell populations, a T-enriched cell population and a B-enriched cell population.

Large numbers of macrophages were not obtainable by the experimental methods used. Only when 7 X 100 cells were transferred were 50% of the recipient mice protected. The question still remains unanswered as to whether more macrophages would convey protection.

Fewer B-enriched spleen cells were needed to transfer protection. As few as 5 X 100 cells transferred solid immunity to trypanosome challenging infections. However, no protection was obtained when as many as 54 X 100 T-enriched spleen cells were transferred.

Spleen cells from mice made hyperimmune to <u>T. rhodesiense</u> by 5 weekly injections of 1 X 10<sup>7</sup> irradiated trypanosomes transferred protection to a trypanosome infection in all recipient mice. It appears that B-enriched spleen cells transfer the protection probably by the production of specific anti-trypanosomal antibody in the recipient mouse. T-enriched spleen cell populations did not transfer protection, while macrophage enriched populations appeared to confer some protection.

## 5. The cynomologus monkey (Macaca fascicularis) as an experimental primate host for visceral leishmaniasis.

Cypomologus monkeys inoculated intravenously with 40-100 million amastigotes of Leishmania donovani (Khartoum strain)/kg body weight invariably developed a clinical illness similar to that observed in visceral leishmaniasis of man. The initial clinical sign usually was splenomegaly in 3-7 weeks post-exposure followed shortly by the onset of pancytopenia. Typical changes included a drop in platelet count from 5-600,000 to 100,000/mm³ or less, a decrease in packed cell volume from 32-40% to 7-17%, a drop in total white count from 5-10,000 to 1-3,000/mm³, and weight loss averaging 15%. Animals usually died within 6-20 weeks post-exposure. At necropsy spleens and livers from infected animals averaged 8.5 and 2.5 times heavier, respectively, than those organs from non-infected control monkeys. Parasites were seen microscopically in liver, spleen, and bone marrow at necropsy. Promastigotes could be cultured from bone marrow using Tobie's diphasic, Tanabe's, and Mansour's media. Histopathologic lesions included severe diffuse granulomatous inflammation of spleen, liver,

and abdominal lymph nodes. Granulomatous interstitial pneumonia and epididymitis also were evident. Meningeal hemorrhages occasionally were observed. These results suggest that this monkey model may be suitable for use in studies relating to the biology, immunology, and pathogenesis of this disease as well as to the testing of anti-leishmanial compounds.

### 6. Microfilariae found in dogs in the United States.

Microfilariae found in dogs in the United States should not always be assumed to be those of <u>Dirofilaria immitis</u>, the dog heartworm. Other closely related filarial worms, both in the genus <u>Dirofilaria</u> and in <u>Dipetalonema</u>, also have been detected in dogs. Although these other species cause no apparent pathology, it is nonetheless important to distinguish <u>D. immitis</u> from them before deciding whether or not to subject the canine host to heartworm treatment. This work reports the finding of 4 apparently different species of filarial worms found in dogs in Florida. The presence of multiple filarial worm species probably is not restricted to this particular state. This situation is being used as an example to remind those who work with heartworm of the widespread existence of filariae other than <u>D. immitis</u> that occur in dogs in this country. Emphasis is placed on those morphological characteristics which can be used most practically by the veterinarian in differentiating microfilariae of <u>D. immitis</u> from those of the non-pathogenic species in question.

It has become a common, almost standard, procedure to distinguish between D. immitis and D. reconditum by observing microfilariae from both fresh blood and from the sediment of a Knott's preparation as reviewed by Jackson and Otto. These procedures were followed in the present study. Additional methods included placing methylene blue-stained Knott sediment onto a microscope slide and photographing with either bright field or phase light. Photographs were taken with a Zeiss ULTRAPHOT II photomicroscope. Measurements were made with a calibrated ocular micrometer. These procedures also were followed in the preparation and description of the other forms of microfilariae discussed in this work unless otherwise specified.

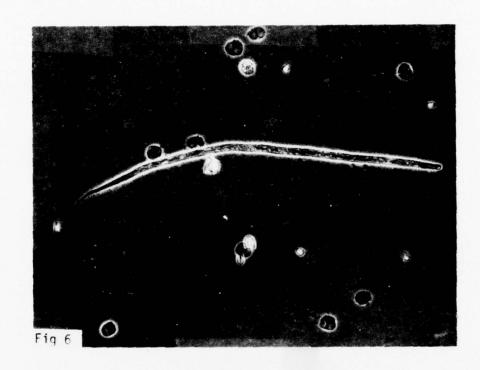
The second procedure used in this work, although not appropriate as a routine method, may rather easily be utilized when special needs arise. This hematoxylin method utilized microfilariae from Knott sediment and resulted in permanently stained and mounted specimens. Sediment was smeared onto a slide, allowed to air dry, and then fixed for 10 minutes in equal parts of 95% ethanol and ether. Slides were air dried and then stained in Delafield's hematoxylin for 1 hour. Specimens were rinsed briefly in 0.05% HCl and then in running tap water until blue color appeared, then air dried, and coverslipped with diaphane. These specimens were observed and photographed with bright field light.

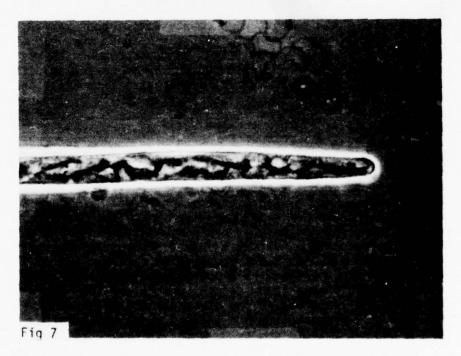
The most frequently encountered problem in differential diagnosis is between microfilariae of <u>Dirofilaria immitis</u> and those of <u>Dipetalonema</u> reconditum, also a common parasite of dogs in the United States. Numerous

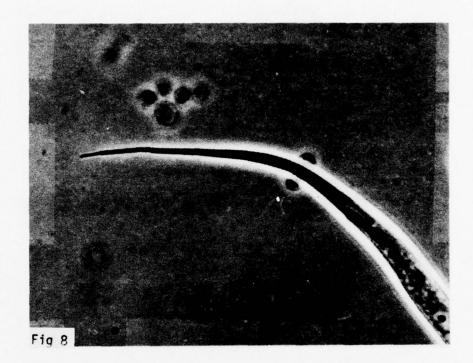
characteristics may be used to differentiate these 2 species. Microfilariae of D. immitis usually are numerous in whole blood while those of D. reconditum commonly are few. D. reconditum microfilariae in general show progressive movement in a wet mount while those of D. immitis exhibit stationary movement. It should be noted, however, that D. immitis may show rather slow progressive movement if observed in blood which is less than 1 hour old while D. reconditum may show periods of rapid progressive motion interspersed with periods of rather stationary movement. A whole microfilaria of D. immitis and D. reconditum from a Knott preparation are shown on low power in Figure 6 and 12, respectively. Two distinguishing characters are readily apparent at this magnification: 1) D. immitis is a larger species which is both longer and wider than D. reconditum; and 2) D. immitis has a rather straight body while that of D. reconditum is curved. Other differentiating characters become apparent when these 2 species are observed with oil emersion. The cephalic or head end of D. immitis (Figure 7) is tapered while that of  $\underline{D}$ . reconditum (Figure 13) ends bluntly, similar to the end of a broomstick. The tail or caudal end of these 2 species usually is strikingly different with D. immitis (Figure 8) showing a straight tail while  $\underline{D}$ . reconditum (Figure 14) has a curved or button-hooked tail. In our experience, D. reconditum showed a curved or button-hooked tail in only about 75% of the specimens examined. We called a tail as being curved if it was bent at least 45°. D. immitis consistently showed a straight or only slightly bent tail.

The next 6 figures show microfilariae which are stained using the hematoxylin method. The distinguishing characters of body length, width, and general shape of <u>D. immitis</u> and <u>D. reconditum</u> again are evident on low power (Figure 9 and <u>15</u>, respectively). When observed with oil emersion, these 2 species exhibit characteristics which may be useful in differentiation which are not obvious in microfilariae taken from Knott sediment. Since the body nuclei are distinctly stained, the length of the caudal space behind the posterior-most nucleus may easily be measured. This space averages 18% longer in D. immitis (Figure 10) than in D. reconditum (Figure 16). Another useful character is the presence of annular striations. These are most obvious in the caudal space of D. immitis (Figure 10) although they are present throughout the entire length of the microfilaria. The caudal space of D. reconditum showing the lack of striations is pictured in Figure 16. The presence of the hooked tail in D. reconditum (Figure 16) and the absence of this character in D. immitis (Figure 10) is easily seen. The cephalic ends of hematoxylin-stained D. immitis (Figure 11) and D. reconditum (Figure 17) also show useful characteristics which are not visible with the Knott preparation. The column of body nuclei and the cephalic space are easily seen with the latter averaging 1.7% longer in D. immitis than in D. reconditum. Also, the annular striations in D. immitis are obvious. The distinction of tapered versus blunt cephalic end in these 2 species is not evident using hematoxylin. Both usually appear to have bulbous heads.

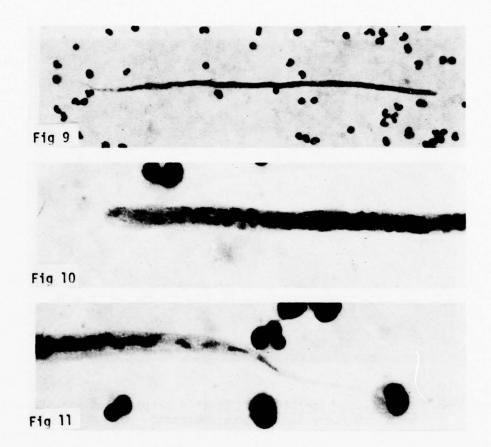
A third microfilaria found in dogs initially was discovered in Florida in May, 1973. This species is evidently also a normal parasite of dogs







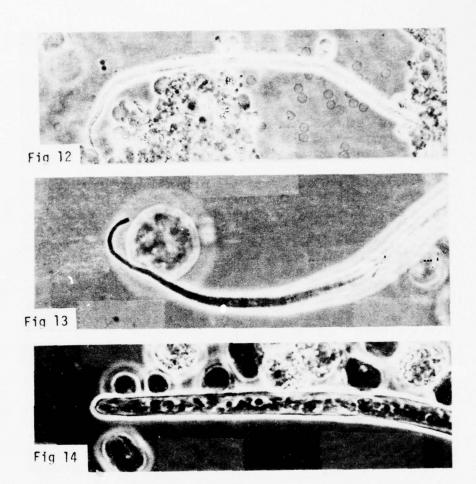
Figures 6-8. Dirofilaria immitis from Knott's preparation and stained with methylene blue (phase contrast). 6. Whole microfilaria showing a straight contour (250 X). 7. Tapered cephalic end of microfilaria (1000 X). 8. Caudal end of microfilaria with straight tail (1000 X).



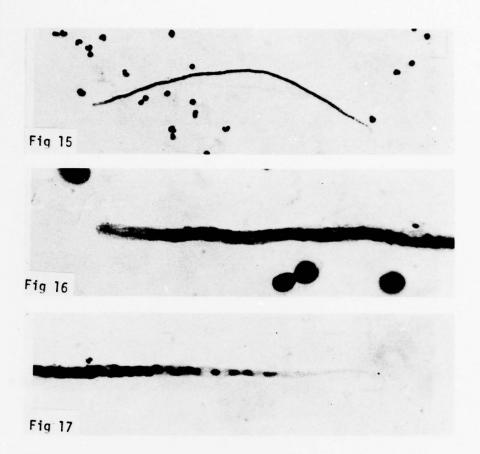
Figures 9-11. Dirofilaria immitis from Knott's preparation and stained with Delafield's hematoxylin. 9. Whole microfilaria with straight contour (250 X). 10. Caudal end of microfilaria showing a straight tail. Annular striations are pronounced, particularly in the caudal space behind the last tail nucleus (1000 X). 11. Cephalic end of microfilaria showing cephalic space in front of anteriormost nuclei. Annular striations are present but indistinct (1000 X).

and appears to belong to the genus Dipetalonema. Because no necropsies of infected dogs could be performed, the adult has not been found. Therefore, a species designation cannot be made at this time Dr. R.F. Jackson found this new worm species in 4 greyhounds which had been referred to him for heartworm treatment. Actually, these 4 animals were only part of a larger group of 12 greyhounds that were referred to him for chemotherapy. The other 8 dogs were native to the United States. Upon repeat blood examination, R.F.J. found what appeared to be 3 different forms of microfilariae. The native U.S. dogs were positive for either D. immitis or D. reconditum or had mixed infections of these 2 species. It was obvious, however, that the microfilariae from the Irish dogs were smaller than either of the other 2 species and did not possess a button-hooked tail. The imported greyhounds had been born and raised in Ireland and shipped to the U.S. when they were about 1 year old. The discovery of microfilaremias took place 6 months later. All 4 dogs were followed for ever 2 years until they eventually lost their infections. During this period, they were housed in various kennels with numerous other greyhounds, none of which ever demonstrated similar microfilariae in their blood. In April, 1975, a fifth greyhound was found with this new filarial worm by Dr. Jackson just 4 days after it had been shipped to Florida from Ireland. In May 1975, Dr. Jackson travelled to Ireland where he visited 2 kneels which had been the source of the dogs which were found positive in Florida. He found that none of 8 dogs examined were infected in one kennel but 2 of 5 dogs were infected in the second kennel. The results of these findings and the earlier cases diagnosed in Florida which included a detailed morphological description of the microfilariae were reported by Jackson et al. This Irish filaria is termed Dipetalonema sp. for the purposes of this paper and will be compared with Dipetalonema reconditum which it closely resembles. Both species usually are found in small numbers in dog blood and show progressive movement in fresh blood. Each species exhibits a blunt, broomstick-like head but Dipetalonema sp. shows a straight tail in contrast to the hooked tail of D. reconditum. No specimens from Knott sediment were available for photographing. A hematoxylin stained microfilaria of Dipetalonema sp. is shown in Figure 18 which demonstrates the typically straight body in contrast to the curved body of D. reconditum (Figure 15). Body length of Dipetalonema sp. averages 7% less than that of D. reconditum. Likewise, body width is somewhat less. Dipetalonema sp. shows a somewhat longer cephalic space (Figure 19) than D. reconditum (Figure 17). The caudal space of Dipetalonema sp. (Figure 20) is straight and is slightly shorter than that of D. reconditum (Figure 16). Neither species shows annular striations.

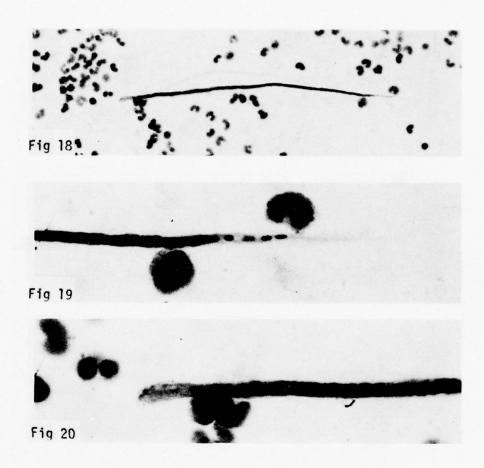
Microfilariae of what appears to be a fourth species now have been found by Dr. Jackson in his practicle. These specimens which were recovered from 2 native U.S. dogs are strikingly larger than microfilariae of D. immitis. Unfortunately, neither dog could be necropsied so adult worms have been unavailable for study. The microfilariae are found in relatively few numbers in fresh blood and exhibit stationary movement. Specimens from Knott sediment average 17% greater than the length of D. immitis. They



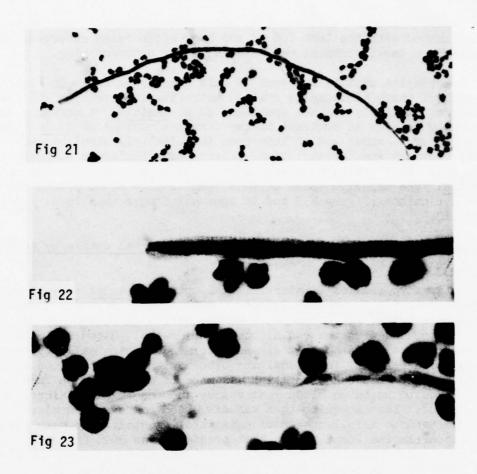
Figures 12-14. Dipetalonema reconditum from Knott's preparation and stained with methylene blue (phase contrast). 12. Whole microfilaria showing a curved contour (250 X). 13. Blunt cephalic end of microfilaria (1000 X). 14. Caudal end of microfilaria with button-hooked tail (1000 X).



Figures 15-17. Dipetalonema reconditum from Knott's preparation and stained with Delafield's hematoxylin. 15. Whole microfilaria with curved body (250 X). 16. Caudal end of microfilaria showing button-hooked tail. Annular striations are not seen (1000 X). 17. Cephalic end of microfilaria showing the clear cephalic space (1000 X).



Figures 18-20. Dipetalonema sp. from Knott's preparation and stained with Delafield's hematoxylin. 18. Whole microfilaria showing a straight contour (250 X). 19. Cephalic end of microfilaria showing clear cephalic space (1000 X). 20. Caudal end of microfilaria with a straight tail. Annular striations are not evident (1000 X).



Figures 21-23. Unknown species from Knott's preparation and stained with Delafield's hematoxylin. 21. Whole microfilaria showing a curved body (250 X). 22. Caudal end of microfilaria with a straight tail and prominent annular striations (1000 X). 23. Cephalic end of microfilaria with annular striations evident in the clear cephalic space (1000 X).

show a tapered head and a straight tail. The body shape is markedly crescent shaped with the last 20% of the body often being recurved. No specimens from Knott sediment were available for photographing.

A hematoxylin stained specimen of this species is shown in Figure 2. The length of these hematoxylin stained microfilariae averages 18% greater than that of similarly stained specimens of D. immitis. A markedly curved body usually is seen in contrast to the straight contour of D. immitis (Figure 9). The caudal end of this form is relatively straight and exhibits distinct annular striations (Figure 22) similar to D. immitis (Figure 10). This space, however, averages 45% longer than that seen in D. immitis. The cephalic space of this species also shows pronounced annular striations (Figure 23) and is somewhat longer than in D. immitis (Figure 11).

### 7. Filariasis: Quantitative studies of microfilarial uptake by Aedes aegypti.

Quantitative studies of microfilarial uptake by Aedes aegypti which were exposed to Brugia pahangi-infected hosts were performed. Mosquitoes were fed on a patent cat which had been inoculated intravenously 30 minutes beforehand with the radioisotope Cerium-144. Blood volume uptake of 40 randomly selected mosquitoes was determined isotopically by comparing gamma emission rates of individual mosquitoes with those of blood aliquots from the infected cat. A prediction was made of microfilariae ingested by each mosquito based on blood meal volume and the level of microfilaremia in host blood. Each mosquito then was dissected and exam ned microscopically to determine actual number of parasites ingested. The mean percentage of microfilariae found versus that predicted was 30% (range = 0-1000%). Data from a second similarly designed experiment using another cat yielded a mean percentage of actual versus predicted parasite uptake of 201% (range = 14-665%). Results previously reported by us on membrane blood feeding studies using Ce-144 had suggest that A. aegypti neither concentrated nor excluded microfilariae during feeding. In these initial studies, mean percentage of actual versus predicted parasite uptake was 83% (range = 38-166%). Data from the present work demonstrated a greater range of microfilarial uptake than that seen in the membrane feeding studies. Also, mosquitoes on the average ingested significantly greater numbers of parasites than expected. These findings were the result of heterogenous distribution of microfilariae in host blood. Significantly more predictable parasite uptake levels were obtained when mosquitoes were fed from a membrane feeder.

### 8. Quantitative changes in astrocytes after portacaval shunting in chimpanzees and in a human patient with intact liver parenchyma.

Alzheimer II astrocytes are a prominent histopathological feature of the encephalopathy associated with human cirrhosis and with other liver metabolic disorders. Recent investigations have shown that similar astrocytic changes can also be produced in a variety of animal models where hyperammonemia has been induced either chemically or by construction of a portacaval shunt. Rarely, clinical encephalopathy and Alzheimer II changes have been found in human patients in which portacaval shunt was performed without significant accompanying liver disease. Analysis of such a case and an appropriate control led us to examine the brains of non-cirrhotic experimental and control chimpanzees who received portacaval shunts in the course of a study of hepatosplenic schistosomiasis. In that condition, there is persistent presinusoidal portal hypertension, but liver cell function is only terminally affected in either humans or in experimental chimpanzees. Portacaval shunt operations for the relief of schistosomal portal hypertension in ednemic patients have been largely abandoned because of a high incidence of encephalopathic sequelae.

Most pathological studies of "Hepatic encephalopathy" have failed to measure the quantitative extent of the histopathological glial changes. Those quantitative studies which have been done in man, and in the rat model have been mainly concerned with the degree of astrocytic changes rather than with topographic distribution or their relationship to clinical and metabolic alterations or time course. We have studied the distribution of histological changes in different regions of the brains in a portacaval shunted human case and in portacaval shunted chimpanzees. Correlations of these changes to clinical illness, duration of shunting, and blood ammonia levels have been analyzed.

#### Human Cases.

# Case Report, PBBH #A72-286 (Portacaval shunt):

A 69-year-old white male, former automobile mechanic, was first admitted to another hospital 12 years antemortem for evaluation of hematemesis. The clinical diagnosis at that time was severe alcoholic cirrhosis with esophageal varices. An end-to-side portacaval shunt was constructed, presumably to relieve "dilated gastric veins". The surgeons noted no splenomegaly, and an intraoperative liver biopsy revealed only minimal fibrosis. Liver function tests were normal.

Episodes of confusion, impairment of memory, visual blurring, ataxia, and bilateral lower face twitching were first noted by the patient one to two years after operation of his shunt. These "spells" were precipitated by high meat or protein intake and were avoidable by dietary restriction. Six years post-shunt, neurological evaluation at a second hospital showed the patient alert but vague with slow responses. He had been neglecting his diet guidelines. His ability to form new associations was poor and his fund of knowledge was fair. He showed asterixis of hands and feet; symmetrical, markedly hyperactive deep tendon reflexes, cerebellar ataxia, and myoclonic jerks. No telangiectasis was noted in the skin, and the liver was normalized. No Keyser-Fleischer rings were found and ceruloplasmin levels were within normal limits. Pertinent laboratory values indicated normal liever function. Serum ammonium, however, was markedly elevated with values greater than 150  $\mu$ g%. EEG showed bitemporal theta activity. He was subsequently maintained on oral neomycin, with

exacerbation of symptoms every time the regimen was neglected.

Then days prior to death, the patient was first admitted to the Peter Bent Brigham Hospital with a 25% third degree burn of the trunk and upper extremity. Previous neurological findings were confirmed. The patient died of shock and bronchopneumonia with gram-negative sepsis.

A general autopsy showed the liver to be normal except for very scant periportal fibrosis. Neuropathological findings will be described below.

### Control case: PBBH #A73-62

This patient had chronic renal failure and hypertension. Autopsy showed end stage kidney disease and pyelonephritis. Neuropathological examination was normal except for athero- and arteriolar sclerosis.

#### CHIMPANZEES:

Six of eight young West-African chimpanzees were subjected to an end-to-side portacaval shunt eight weeks after experimental infection with Schistosoma japonicum, as more fully reported elsewhere. Animals with neurological impariment after shunting received a low protein diet. Routine observations showed that three of the animals demonstrated some neurological dysfunction although detailed serial neurological examinations were not done. These clinical symptoms were: lethargy, weakness, bellowing, anorexia for #618, weakness, bellowing, absence of palpebral and corneal reflexes for #761, and weakness, ataxia, lethargy for #376. One chimpanzee, #L-364, with hypertrophied astrocytes and elevated ammonia levels showed no neurological alteration to casual observation. Blood ammonia levels of these animals were elevated

The animals were sacrificed at 11 and 30 weeks post-shunting with the exception of chimp #618 who became extremely ill and was sacrificed at seven weeks post-shunting.

At necropsy, the livers of the experimental chimpanzees were examined microscopically and were judged to be normal, except for moderate number of schistosome granulomas. Mild pipe-stem fibrosis was noted in only one case, #204. Four control chimpanzee brains of young animals were obtained from the National Institutes of Health. Liver specimens from the control chimps were not available.

#### Histopathological Studies

Four of the six portacaval-shunted chimpanzees' brains were first studied qualitatively. One of these, #618, was then chosen for detailed quantitative study since that animal had exhibited typical neurological impairment and histopathologically showed representative changes. A control chimpanzee brain was similarly quantitated. The single human

portacaval shunt case was also quantitatively studied compared with chimpanzee brain #618, as well as with chimpanzee and human control brains.

Small, medium, and whole-brain paraffin sections were cut at  $12\mu$  and stained with hematoxylin-eosin and special stains. All quantitative observations were made on H & E-stained giant sections. Subjective evaluation of Alzheimer II change was performed "blindly" by two independent observers, and was later correlated with the clinical observations.

Nuclei were classified as astrocytic when they conformed to all of the following criteria: a) Absence of visible cytoplasm, b) Absence of prominent nucleolus, c) No prominence of dense, intensely basophilic chromatin, as is seen in oligodendrocytes, and d) A tendency toward marginations of the existing chromatin. It should be noted that nuclear size, per se, was not a criterion for inclusion or exclusion. The presence of a pronounced intranuclear glycogen dot is generally regarded as typical of Alzheimer II astrocytes, and though this feature is not prominent in H & E stains, checks of PAS-stained sections showed it to be present in many nuclei in our material.

The degree of astrocyte hyperplasia and nuclear enlargement was assessed in coronal sections of four gray and white matter areas: insular cortex, caudate nucleus, medial globus pallidus and the centrum semiovale. High-power fields (450 X) of 405µ diameter were chosen randomly. Care was taken to sample equally all regions. The number of astrocytes was determ ned in 20 random fields for each area. In every fourth field, the longest and shortest diameters of astrocyte nuclei were measured with an ocular micrometer. These counts were performed by two independent observers and practice counts indicated 90% agreement between the two. Care was also taken not to bias these measurements toward larger nuclear profiles, and at least 60% of the nuclei were measured in each of the 64 fields studied. Following the methods of Gutierrez and Norenberg, volumes were calculated using the formula for a prolate spheroid,  $/6~\rm LS^2$ , where L and S are the longest and shortest diameters, respectively. Abercrombie's formula was used to correct these figures for the dependence of apparent incidence of enlarged nuclei on section thickness.

The statistical significance of the data was evaluated using Student's t-test.

RESULTS

Neurological examination:

(A) Human cases:

The portacaval shunt patient's brain PBBH #A72-286, weighed 1,260 gm.

Grossly, it was normal except for a brown-yellow line of discoloration in coronal sections between the cortex and underlying white matter.

Microscopically, prominent hyperplasia and hypertrophy of protoplasmic astrocytes was observed, these cells being identified as astrocytes based on the criteria given in the Histopathological Studies section. Sometimes their nucleus appeared lobulated and occasionally they occurred in pairs. The cerebral cortex, basal ganglia, brainstem and Bergmann cell layer all showed astrocytic changes.

In some regions, particularly in the depth of cortical sulci, there was status spongiosus, slight increase in cellularity, and prominent capillaries. Status spongiosus was also observed in the caudate nucleus, putamen, and globus pallidus.

The control human brain, PBBH #A73-62, showed no evidence of hyperplasia or hypertrophy of astrocytes.

#### (B) Chimpanzee brains:

Grossly, the brains appeared normal. Histologically, the astrocytic nuclei were strikingly swollen, often vesicular, and were distributed throughout the cerebral cortex, basal ganglia and pons. In animal #761, they were also prominent in the cerebellum. No astrocytic mitoses were observed. Neuronal loss was not noticed.

The chimpanzee control brains were normal grossly and microscopically.

#### Quantitative studies

Overall, Abercrombie-corrected data showed a 26% increase of astrocytic nuclei for the shunted chimpanzee brain #618 as compared to 41% increase in the shunted human case, PBBH A72-286. With regard to the four brain regions sampled, the shunted chimpanzee showed an increase of 25% in the insula, 17% for the caudate nucleus, 77% in the medial globus pallidus, and a 17% decrease in the centrum semiovale. The shunted human case showed an increase of 45% in the insula, 16% in the caudate, 33% in the medial globus pallidus, and a 70% increase in the centrum semiovale.

This study shows that an "Alzheimer II" astrocytosis, namely an increase in the number and size of astrocyte nuclei, is produced in the chimpanzee after portacaval shunting. It also confirms the occasional reports of encephalopathy and Alzheimer II changes in human brains after portacaval anastomosis even in the absence of significant liver disease.

Various animal models have been developed to study "hepatic encephalopathy". To our knowledge, this is the first report of a chimpanzee model of "hepatic encephalopathy" or, more accurately, portacaval shunt encephalopathy. By far the most commonly used model has been that of the rat.

In comparing the human encephalopathy with the chimpanzee's encephalopathy, there is good agreement.

The morphological changes in the chimpanzees appear quite similar if not identical to the pathological changes in human cases. The chimpanzees also seem to show a correlation between their morphological changes and clinical neurological alterations. It is debatable whether the rat model shows neurological alterations similar to the features of the human encephalopathy.

Some investigators have suggested that hypertrophy of astrocytic nuclei is largely an artifact of how the brain is fixed. Immersion fixation produces nuclear swelling as compared to perfusion fixation. Gutierrez and Norenberg also mention preliminary evidence that processing tissues through a series of alcohols contributes to this phenomenon. Using a rat hepatic encephalopathy model Norenberg was unable to demonstrate enlargement or hypertrophy of astrocytic nuclei in stained frozen sections of brain of decapitated animals. He considered astrocytic nuclear hypertrophy to be an artifact.

The striking dissimilarity found in this study between the volume enlargement for the chimpanzee astrocyte nuclei versus those from the human case may indeed depend upon formalin dilution (10% for the former versus 15% for the latter); the duration of fixation was also not uniform. Because of the possible artifact of swelling, it is the hyperplasia of the astrocytic nuclei rather than the hypertrophy of the nuclei which most interested us.

The primary aim of the present study was to analyze quantitatively the astrocytic changes in terms of regional anatomy--e.g. the insular cortex, caudate, globus pallidus and centrum semiovale.

Our count for astrocytes in four different regions of the brain were based on the quantitative histological techniques described by Gutierrez and Norenberg in the rat model. These authors have discussed the technical difficulties involved.

In our study, the overall increases in astrocyte number were calculated by the Abercrombi formula. The overall values were 26% for the chimpanzee and 41% for the human. With the possible exception of the chimpanzee globus pallidus, these were not strikingly (i.e., several fold) different from the 33% Abercrombi-corrected increase in astrocyte number found by Gutierrez and Norenberg in the rat. It should be noted that in those animals hyperplasia of the astrocytes were produced within 7 1/2 hours after injection of methionine sulfoximine. That such a proliferation could occur so rapidly is remarkable, and should be further investigated with other models or methods.

Hyperplasia of astrocytes was found to be highly significant in all of the gray matter regions examined, except for the centrum semiovale, in the shunted chimpanzee, a finding that may require further confirmation.

As to the human brain, it showed not only significant astrocyte hyperplasia in the three gray matter regions examined but also in the centrum semiovale. Possibly, the much longer duration of shunting in the human case accounts for the development of true astrocytic hyperplasia in white as well as gray matter. A less likely possibility is that species differences play a role. Further study is needed to elucidate these points.

In most investigations, ammonia metabolism has been clearly implicated as a prime factor in eliciting encephalopathy. Regarding the duration of the hyperammonemia, none of the studies now in the literature has directly investigated this problem in different animal species. In this study we did not find a good correlation between the duration of portacaval shunting in the chimpanzees and the degree of astrocytic hyperplasia. However, only three ammonia determinations were done during the clinical course of the animals in our series. More frequent determination could be done in future studies in order to be sure of this point.

The duration of portacaval shunting for the human case in this study was more than 12 years, and encephalopathy had been present for more than 10 years. That the astrocyte hyperplasia was not more marked in this material argues for the view that the ability of astrocytes to respond (at least morphologically) to hyperammonemia is fluctuant and limited. This view fits with the finding of Norenberg and Lapham that astrocytes of the rat cerebral cortex in the terminal stages of encephalopathy show degenerative changes at the ultrastructural level.

## 9. Experimental Schistosoma japonicum infection miniature pigs.

Our knowledge of the pathogenesis of pipestem fibrosis and renal lesions occurring in man as a result of infection with Schistosoma japonicum has been hindered by the lack of a suitable animal model in which these lesions can be reproduced. The chimpanzee is the only such model which presents a disease similar in most respects to that observed in man. Yet the severe limitations imposed by the low availability and the high costs of purchasing, housing and handling chimpanzees make it necessary to continue our search for a suitable host which may serve as an additional model of human disease. In view of the physiological similarities between procines and humans, and of the possible role of pigs in the transmission of S. japonicum, it was reasonable to attempt an evaluation of the susceptibility of miniature pigs to schistosome infection.

Eight 10-week old pigs (nos. 5, 6, 7, 8, 10, 11, 12 and 13), ranging in weights from 8 to 11 kg, were each exposed percutaneously to the Japanese strain of S. japonicum (40 cercariae per kg body weight). Cercarie were obtained from freshly crushed Oncomelania hupensis nosophora snails and applied to the clipped groin area of the anesthetized pig. All pigs had been born and weaned on an experimental research farm and were in good general health, although all animals harbored natural

Table I. Exposure and infection data on S. japonicum in miniature pigs.

D		Weigh	nt (kg)		First	0		TEDOT) *	No	o. worms	recovered	
Duration of infection (weeks)	Pig no.	At expo- sure	At Nec- ropsy	No. of cer- cariae	fecal eggs detected (week)	Liver	gg assay (? Small intestine	Large intestine	Mature males	Mature fe- males	Stunted, sex undet.	Total
9	12	10	18	400	7	427	1	48	5	13	33	61
22	8	10	26	400	8	19	16	27	0	0	0	0
	13	9	23	360	8	276	4	422	6	3	0	9
23	5	11	16	440	12	ND†	ND	ND	ND	ND	ND	ND
	10	9	21	360	7	ND	ND	ND	ND	ND	ND	ND
45	7	11	28	440	7	157	79	295	1	0	23	24
	11	11	29	440	7	51	191	88	0	0	0	0
55	6	8	29	320	None	9	14	37	0	0	0	0

\* NEPGT = No. of eggs per gram of tissue (KOH digestion).

† ND = Not done.

Balantidium coli infections and one animal (No. 6) presented unfertilized Ascaris ova in the feces. Weekly fecal examinations were initiated 5 weeks post-exposure and attempts were made to quantitate fecal egg excretion in terms of numbers of eggs per gram of feces (NEPGF) after concentration by the formalin-ether-buffered alcohol technique. Adult worms were recovered by perfusion at various times post-exposure (Table V) and tissues were obtained for histopathologic examination and organ egg assay by the KOH digestion method of Cheever.

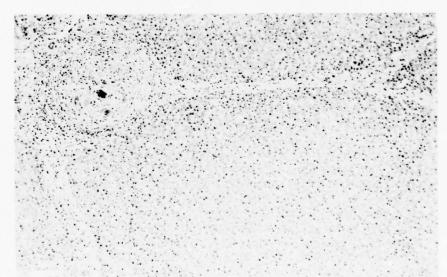


Fig 24

Figure 24. Pig No. 12, 9 weeks post-exposure. Single egg granuloma with intact meracidium and scattered lymphoid cell infiltrate.

The liver parenchyma shows a normally septate pattern (H&E, X110).

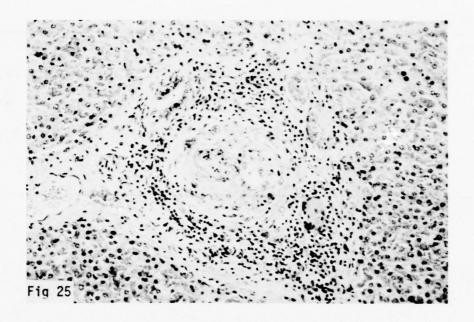


Figure 25. Pig No. 5, 23 weeks post exposure. Involuting granuloma containing egg shell remnants and mild concentric fibrosis. Note bile duct inflammation (arrows) and severe venous and sinusoidal congestion (H&E, X300).

The parasitologic data accumulated (Table V) generally indicates difficulty in establishing acute S. japonicum infections in pigs. Although some tissue egg deposition had occurred in all of 6 animals so analyzed, no worms were recovered by extensive perfusion and autopsy of 3 of these pigs. Concurrently, 7 of 8 pigs presented eggs in fecal specimens but in none of these did the counts go above 4 EPGF and most pigs presented negative stools after 14 weeks post-exposure.

On pathologic evaluation gross lesions were inconspicuous in all of the animals. Histologically, all animals showed evidence of active but generally mild infection with a further apparent decrease in activity and intensity by 45 to 55 weeks post-exposure. One large granuloma suggestive of a dead-worm focus was seen at 22 weeks (No. 13). Whereas immature and mature eggs predominated in tissues obtained at 9 weeks (No. 12) (Figure 24), degenerated and calcified forms abounded in all later specimens (Figure 25). Few eggs were seen in sections of small intestine; the colon showed patchy, active areas of egg deposition with mild inflammation and only a few granulomas confined to the mucosa and submucosa. Small liver granulomas containing single eggs (only about 1 of 10 granulomas contained egg clusters) were seen mainly in the portal spaces. Macrophages and fibroblasts were numerous in these granulomas; few neutrophils were present. "Pseudoabscesses" and

Hoeppli phenomena were not seen and histiogranulocytic granulomas were very rare. Involution of granulomas occurred either with (Figure 25) or without mild focal residual fibrosis. Focal inflammation was minor in all cases and significant parenchymal lesions, or pipe-stem fibrosis, were not seen. Only sporadic granulomas appeared in the lungs. The spleen showed some brown pigment in macrophages, and the abdominal lymph nodes showed reticular proliferation and an increased eosinophilia. No schistosoma lesions were detected in samples of other organs (heart, esophagus, urinary bladder, pancreas or ovary).

Non-schistosoma lesions included: active, mild cholangitis with minimal portal fibrosis and marked abdominal congestion (No. 5; this animal died unexpectedly following a short period of lethargy and anorexia and probably had an enteric bacterial superinfection); and mild focal interstitial nephritis (No. 13).

The manifestations of schistosomiasis japonica in the miniature pig turned out to be minimal. The delayed appearance of eggs in the feces, the resultant extremely low egg counts, the rapid reversion to negative fecals and the few stunted worms finally recovered dispel this animal as a model host on parasitologic grounds. With regard to pathology, the lesions of acute "toxemic" or of severe chronic infections in man were not reproduced; specifically, no evidence of pipestem fibrosis was found. The mild degree of infection and the modest tissue reactions elicited in pigs were reminiscent of those seen in other "hosts of limited susceptibility" described in earlier studies with  $\underline{S}$ . mansoni.

# 10. Radioisotope uptake and retention by cercariae and developing Schistosoma mansoni.

Our recent investigations on the immunology of Schistosoma mansoni infections in the inbred rat have been hampered by the difficulty in differentiating worm subpopulations (immunizing versus challenging exposures) within the same host animal. When dealing with such mixed schistosome populations whose ages are separated by only a few days, morphologic differentiation can be misleading because of varying worm development rates. Radioisotopic labeling of exposure subpopulations may therefore offer a convenient tool for such differentiation of infection subpopulations. In addition, the selective radiolabeling of developing schistosomules might enable more objective evaluations of various metabolic pathways and provide a useful marker to trace the fate of schistosomular products within the host or aid in vitro fractionation of these components. The earlier works of Lewert and Para, Bruce et al. and Para et al. have aptly proven the feasibility of radiolabeling cercariae and following isotope retention/depletion through various periods of development. Our studies were originated as a definitive evaluation of this methodology for identifying selective worm populations and their products for prolonged periods of time.

In Vivo Methodology. The Puerto Rican strains of S. mansoni-infected Biomphalaria glabrata were maintained as previously described. Six weeks following exposure of individual snails to 7 to 10 miracidia each, patency was determined by screening each snail for emerging cercariae. Those snails which were confirmed as positive were isolated in dark pans containing 4 liters of aerated water, each pan containing at least 30 snails. Isotopes were added to the culture water and the snails were maintained in constant contact with this isotope-water medium for the duration of the experiments. Control snails were maintained without isotope.

In an initial experiment 3 isotopes were used: a mixture of 15 purified 3H-L-amino acids (specific activities: 1.23 - 105 Ci per mM of individual amino acid; New England Nuclear); 3H-thymidine (specific activity: 20 Ci per mM; New England Nuclear);  $^{14}\text{C-formate}$  (specific activity: 5.3 mCi per mM; New England Nuclear). Each isotope was added to one snail group in a final concentration of 1.0  $\mu\text{Ci}$  per ml or 0.25  $\mu\text{Ci}$  per ml. Infected snails were allowed to incubate in the presence of isotope for at least 6 days prior to mass shedding for cercariae.

Snails were washed 3 times in dechlorinated water prior to photic release of cercariae. Pools of cercariae thus collected were counted in 0.1 ml aliquots and utilized for (1) rat exposures, (2) schistosomule collection, and/or (3) determination of cercarial uptake of radioisotope. Cercariae were obtained weekly from each snail group for at least 4 weeks to determine the rate of isotope uptake in the intramolluscan system.

Inbred rats (CDF-Charles River Fisher) were exposed to 1000 cercariae each by tail immersion, and the developing schistosomes were recovered by perfusion at various intervals following infection. Schistosomules were obtained following cercarial penetration of a freshly excised, clipped rat abdominal skin membrane and collection in Lactalbumin Hydrolysate-Hanks Balanced Salt Solution, as reviewed by Stirewalt.

Determination of Radioisotopic Incorporation. Radioisotope uptake and retention were determined by liquid scintillation and autoradiographic procedures. Samples of specimens to be subjected to liquid scintillation (cercariae, schistosomules or worms) were washed 3 times in either water or absolute ethanol and counted numbers of specimens from each sample were placed in liquid scintillation vials. In later experiments, specimens were applied to 2.4 cm glass fiber filter discs (Reeve Angel Company) and allowed to dry for at least 24 hours in the dark in NCS tissue solubilizer/ POP toluene based scintillation cocktail prior to counting in a Packard-Tri Carb Liquid Scintillation Counter. As many specimens as possible were incorporated into each sample and radioactivity was calculated in terms of counts per minute (cpm) per specimen or per multiple of specimens. Isotope retention was determined as the per cent retention of radioactivity from that of the original cercarial pool from which schistosomules or developing worms were derived. Values were adjusted for background counts through the subtraction of the isotope incorporation values obtained when

identically treated unlabeled specimens were utilized. From 2 to 6 samples of each specimen pool were used to determine each experimental value.

Radioactivity was also assessed by autoradiographic criteria. Specimens were washed 3 times in absolute ethanol, heat fixed onto precleaned glass microscope slides, washed for 10 minutes in tapwater (16°C), drained, dipped into Kodak NTB-2 liquid emulsion in total darkness, gelled at 4°C, and air dried for 10 minutes in a 37°C vacuum oven. Slides were stored under dessication at 4° for 8 to 24 weeks in a slide box wrapped with light occluding paper. The slides were developed for 4 minutes in Kodak D-19 developer, washed in water for one minute and placed into acid fixer for 4 minutes. The emulsion was scraped from the back of the slides prior to a final 10 minute wash. Specimens were lightly counterstained for 7 minutes in Harris' hematoxylin, washed for an additional 10 minutes, dehydrated in a graded ethanol series, cleared in xylene and cover-slipped with Permount. Specimens were examined by dark-field illumination microscopy to provide a higher contrast to grains of radioactivity.

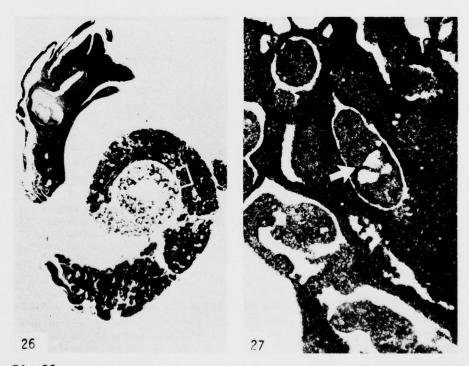


Fig 26 Autoradiograph of a section of *Biomphalaria glabrata* illustrating uniform labeling of snail tissue 3 weeks after the introduction of isotope into the snail culture water. [\*H]-amino acids. Bright field illumination. ×95.

Fig 27 Enlargement of the box area in Fig. 1 showing heavy radiolabeling of intramolluscan schistosoma mansoni (arrow) and surrounding snail hepatopancreatic tissues. Note the absence of isotope in the preacetabular glands of the cercaria. [aH]amino acids. Bright field illumination. ×285.

In Vitro Methodology: Infected rat blood was obtained by cardiac puncture, clotted at 4°C and centrifuged at 5000 g for 30 minutes. The serum thus obtained was applied in a 5 ml volume to a 100 X 2.5 cm Sephadex G-200 column which had been equilibrated with isotonic Tris-PBS (pH 7.2). The sample was then run anti-gravity at a rate of 10 ml per hour at 4°C. The effluent was sequentially chromatographed on a Sepharose 6B column and collected in 5 ml monitored aliquots using a LKB UVICORD III dual beam monitor and fraction collector. The presence of immunoglobulin within these aliquots was determ ned by precipitation with goat-anti-rat immunoglobulin antisera of defined heavy chain specificities. Both gel diffusion and slid phas adsorption confirmatory procedures were utilized. The presence of schistosome antigen was established by the specific inhibition of a preestablished complement fixing antischistosomal antibody/antigen reaction.

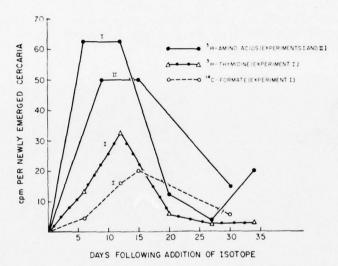
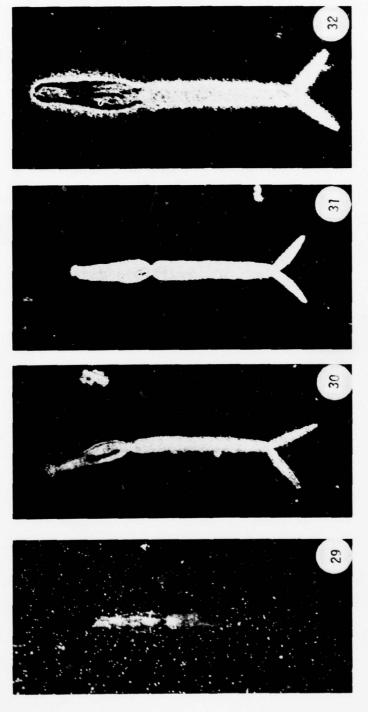


Fig 28 Time-course study of radioisotope uptake by intramolluscan Schistosoma mansoni as determined by liquid scintillation counting of newly emerged cercariae. Positive snails were maintained continuously in culture water containing 1.0  $\mu$ Ci of isotope/ml. Experiment I evaluated the uptake of three different isotopes. Experiment II studied only [\*H]amino acid uptake. Counts (cpm) were adjusted for background radioactivity within control (unlabeled) cercariae.

Radioisotope Uptake. Radiolabeled isotopes were rapidly incorporated into the snail tissues and the developing intramolluscan forms of S. mansoni. This was readily demonstrated by autoradiography of infected snail tissue sections (Figures 26 and 27). The rates of incorporation of radioactivity in newly emerging cercariae are shown in Figure 28. In all cases, the most heavily labeled cercariae appeared to be those which emerged between 6 and 15 days following the addition of isotope. This peak was reached the earliest (6 to 9) days by cercariae incorporating 3H-L-amino acids at a concentration of 1.0 mCi per liter of culture water. Additionally, these organisms demonstrated the highest peak levels of radioactivity (50 to 63 cpm per carcaria). Two representative experi-



F1qs 29-32 Autoradiographs of newly emerged Schistosoma mansoni cercariae collected 10 days after the introduction of radioisotope to the infected snail culture water and stored for 8 weeks prior to developing. Dark field illumination. ×155, Fig.29 Unlabeled cercaria. Note the scattered grains of background radioactivity. Fig.30 [\*H]amino acids-labeled cercaria. Fig.31 [\*H[thymidine-labeled cercaria. Fig.32 [\*C]formate-labeled cercaria. The dark streaks in this figure and in Fig.30 represent internal unlabeled areas as accentuated by the focal planes of the photographs.

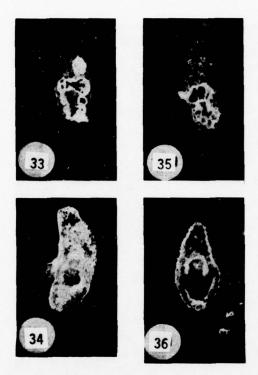
mental exposure groups are shown. In contrast, cercariae exposed to 3H-thymidine or <sup>14</sup>C-formate exhibited relatively lower radioactivity incorporation levels (peaks of 32 and 20 cpm per cercaria respectively). Regardless of the isotope used, the peak radioactivity levels were followed by a rapid decrease of cercarial labeling. Counts for cercariae obtained 25 to 30 days following isotope introduction to infected snails represented only 6 to 30 per cent of the peak levels obtained at days 6 and 15.

Whereas scintillation procedures revealed quantitative differences in isotope uptake, autoradiographs of newly emerged cercariae dramatically demonstrated qualitative morphologic incorporation of radiolabel (Figures 29-32). All of the cercariae from snails incubated with any of the three isotopes were intensely labeled.

Radioisotope Retention. Figure 38 illustrates the liquid scintillation retention pattern of 3H-L-amino acids following cercarial penetration and during development in the rat host. Approximately 50 per cent of the label was retained in schistosomules obtained immediately after in vitro penetration of rat abdominal skin membranes. The majority of this depletion probably can be attributed to tail loss during penetration since radioautography shows heavy labeling in this area. Worms derived from the same cercarial pool and obtained by interval perfusion of different rat groups demonstrated a marked and rapid loss of isotope during early worm development. By day 19 post-exposure, less than 1 per cent of the original cercarial label had been retained. Additionally, this value was indistinguishable either from background counts or from unlabeled worms of the same age.

Autoradiography provided the sensitivity necessary for revealing low radioactivity levels not detectable by liquid scintillation criteria. Whereas counts could not be obtained after 12 to 19 days post-exposure, autoradiographs examined by dark field illumination showed distinct retention and localization of isotope after 3 weeks of worm development (Figures 34-37). As with labeled cercariae, there were qualitative differences in the patterns of localization of radiolabel in the schistosomules. <sup>3</sup>H-amino acids showed heavily confluent localizations (Figure 36). In the case of <sup>3</sup>H-amino acids, this pattern of radiolabeling could be obtained for as long as 6 weeks of worm development (Figure 37).

Effects of Radioisotope on Biological Parameters. The first question to be asked was whether the presence of the radioactive label appeared to have any adverse effects upon the developing snails, cercariae or schistosomules. Accordingly, the effects of radiolabeling upon schistosome development were evaluated by 3 basic criteria (Table VI): snail mortality, cercarial production and cercarial penetration capabilities in an in vitro system (rat abdominal skin). In general, values so obtained were within normal limits based upon unlabeled control values and upon our prior experience with the S. mansoni life cycle maintenance system. In addition, morphologic worm development in, and recoveries from, mice and rats exposed to these cercariae were comparable with those obtained



Figs 33-36 Autoradiographs of 3-week-old Schistosoma mansoni from CDF rats which were exposed to cercariae from the same respective pools as those cercariae depicted in Figs. 4-7. Worms were recovered by perfusion 3 weeks later. Slides were stored for 10 weeks prior to developing. Note that with dark field illumination the parasite gut is depicted as white but does not imply concentrations of radioactivity. This was confirmed by careful bright field observations. Fig. 33 unlabeled schistosome. Fig. 34 [34]amino acid-labeled schistosome. Fig. 35 [34]thymidine-labeled schistosome. Fig. 36 [34]fromate-labeled schistosome.

in the numerous normal infections which we have maintained for other experiments (data not shown). Indeed, schistosomule recoveries from radiolabeled cercariae tended to be slightly greater than those from unlabeled control groups. It is interesting to note that the maximum cercarial production in labeled snails developed after the peak radioactivity was obtained in cercariae from the same snail groups.

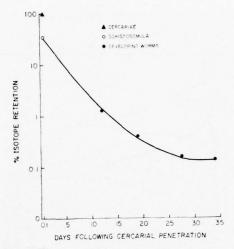
Radioisotopic Fate. When the serum obtained from animals previously exposed to 10,000 3H-amino acids-labeled cercariae was chromatographed, radioactivity was recovered in several discrete areas, corresponding to molecules with molecular weights of >10 to 10 <. Moreover, when these individual peaks were analyzed, multiple components were observed. For example, following acid proprionic column chromatography, the peak corresponding to 7S was found to contain two components: one with a molecular equivalent of 7S which could be precipitated with goat-anti-rat IgG, and another with a molecular equivalent of 4.5S which contained the radioactivity and could competitively inhibit a complement fixation reaction involving schistosomal antigen. That this radioactive profile



Fig 37 Autoradiography of a 6-week-old Schistosoma mansoni labeled with [\*H]amino acids. The procedures were the same as described for Figs. 8-11. Dark field illumination.

was not due simply to radioisotope reincorporation was further shown through the analysis of serum obtained from animals which were exposed to unlabeled schistosomules and free <sup>3</sup>H-amino acids (10X the cpm contained within the <sup>3</sup>H-schistosomules) (Figure 39). A much different and lower distribution of radioactivity was obtained. The 7S component again contained a low molecular weight fraction could inhibit the complement fixation reaction, but which bore no significant radioisotopic label.

Jewsbury and Homewood have previously reviewed the history of in vivo radiolabeling attempts. Their own efforts involved the use of 3H-5-orotic acid to obtain labeled cercariae. Although some initial success in developing labeled cercariae and worms was obtained, analysis of their data is hampered by the small numbers of animals used. A variety of other radioisotopes have been used for specialized experimental purposes, but only two, radioselenium and 14 C-glucose have been directed toward both the intramolluscan uptake and the subsequent retention of isotope by cercariae and developing worms. The earlier work of Cruz, et al., however, first established the feasibility of radiolabel-



Fiq 38 Radioisotope ([\*]amino acids) retention curve for developing *Schistosoma mansoni* in CDF rats. All schistosomula and developing worms were derived from the same cercarial pool. The cercariae were considered to have 100% retention.

ing cercariae (in thic ase by incubating  $\underline{S}$ .  $\underline{japonicum}$  cercariae directly in 59Fe), and tracing the retention of label in worms. Other studies with this isotope have all dealt only with the molluscan uptake and distribution in infected or uninfected B. glabrata.

The primary purpose of our experiments, however, was to develop a system whereby the maximum amount of detectable isotope was incorporated and retained by schistosomes for a significant period of time. We therefore selected isotopes on the basis of their potential for incorporation into relatively stable biosynthetic systems, as well as for ease of handling. By these general criteria one could predict that 3H-thymidine would be incorporated into nucleic acid biosynthesis, 14C-formate into membrane biosynthesis and 3H-amino acids into a variety of protein biosynthetic systems. These distributions were grossly confirmed by the distribution of label within schistosomular autoradiographics. During early schistosome development, however, rapid worm growth and high metabolic activity were apparently such that thymidine and formate were rapidly turned over and eliminated, hence quickly reduced to undetectable levels in the parasite. Our original design was therefore more readily met by theuse of the 3H-amino acid mixture. Analysis of concurrent infections of labeled and unlabeled worm populations in the rat further indicates that no apparent exchange of isotope occurred between the two sub-populations of worms and that a sub-population may be independently followed for an appreciable period of time.

An additional advantage of <sup>3</sup>H-amino acids is that not only were they readily assimilated by intramolluscan developmental forms, but that newly

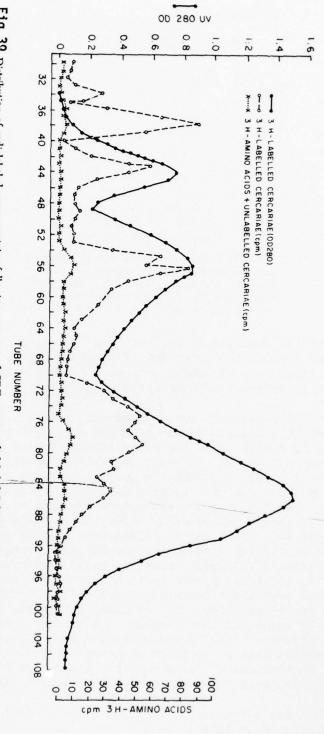


Fig 39 Distribution of radiolabeled serum proteins following exposure of CDF rats to radiolabeled Schistosoma mansoni cercariae. Serum was obtained from unexposed animals, animals exposed to labeled cercariae, and animals exposed to unlabeled cercariae which previously were incubated directly in a [4H]amino acids mixture. The serums were sequentially chromatographed using G-200 and Sepharose 6B sieving gels. The distribution of radioactivity (cpm) and proteins, as measured by absorbance at 280 nm, is depicted.

emerged cercariae obtained during repeated sheddings of the same snail group continued to demonstrate relatively high radioactivity levels. By this economic consideration, therefore, one can make multiple usage of a single isotope administration for different experiments, simply by appropriately timing the schedule of snail shedding. Eventually, however, the isotope is apparently turned over and excreted into the culture water in the form of metabolic by-products which are not readily reincorporated into developing cercariae or are diluted by competing nonlabeled molecules. This is evidenced by the dramatic reduction of label on cercariae shed after 12 to 15 days of continuous snail incubation with isotope.

Of additional critical importance is the apparent lack of effect by the <sup>3</sup>H-amino acids upon snail, cercaria, or schistosomule development. Numerical, functional, and structural analyses have showed normal development by radiolabeled moieties, indicating that their use is biologically reasonable.

Lewert and Para initially proposed the use of radioisotopes for analysis of the fates of worm components. Later studies, such as those of Kusel et al. and unpublished work in our laboratories using NaI 2, have demonstrated the ability to label surface structures of schistosome immunogens and to specifically interact them with antibody. Our present studies clearly indicate that significant amounts of worm-derived material of varying molecular weights can be obtained from the host serum and that at least a portion of these components exist in the form of circulating immune complexes. Moreover, the specific components of these complexes or specific schistosome components derived through in vitro analyses can be uniquely studied using their radioactive label as an identification tracer. Hence, the methodology described here not only enables the long-term simultaneous evaluation of multiple, apparently normal, subpopulations of schistosomes, but also allows the increased efficiency of both in vivo and in vitro analyses of the products of these subpopulations.

#### 11. Morbidity to schistosomiasis in athymic mice.

The principle morbidity of chronic infection by Schistosoma mansoni is the development of hepato-splenic portal hypertension. This phenomenon is apparently related to a granulomatous, cell-mediated, immune response to the schistosome eggs which are deposited in the capillaries of the liver. Indeed, the injection of S. mansoni eggs intravenously can evoke a "pseudotubercle" in the lungs of normal mice.

Warren and his associates have demonstrated that granuloma formation is markedly suppressed in thymectomized chickens. Conversely, bursectomy had little effect. Studies utilizing murine models of granuloma formation are in general consistent with the data obtained from fowl studies in implicating cellular immune mechanisms in granuloma formation. Granuloma formation around eggs which have been injected into previously

infected animals occurs at an accelerated rate when compared to the rate of granuloma formation shown in uninfected animals. This accelerated granuloma formation can be adoptively transferred with cells, but not serum, into syngeneic recipients. A number of in vitro correlates involving a variety of cell-mediated responses to egg immunogen have been developed. Immunosuppressive drugs, thymectomy, heterologous antilymphocyte serum, and antimacrophage serum have all been shown to reduce granuloma formation.

However, recent studies have emphasized the uniqueness of schistosome granuloma formation, since the dominant cell type in this lesion is the eosinophil. Studies of Colley, employing thymectomy, antithymic serum, and cytosuppression in various combinations, did demonstrate that T-cell depletion led to the expected marked reduction in the development of cells which exhibited an anti-soluble egg immunogen mediated blastogenic response in vitro, delayed dermal reactivity, and reaginic antibody response to S. mansoni egg antigen. Moreover, blood eosinophilia was markedly reduced. However, the lesions associated with egg deposition were those of abscess formation with attendant severe host morbidity rather than a simple decrease in granuloma formation.

The congenitally athymic or nude mouse represents an ideal model in which to study murine granuloma formation in response to <u>S. mansoni</u> egg deposition since it represents another model of T-cell immunodeficiency. Although the basic mechanisms whereby the presinusoidal capillary lesions are associated with and/or responsible for the subsequent portal hypertension are unknown, there does exist a general correlation between the severity of granuloma formation and host morbidity. Moreover, granuloma formation is apparently related to an eosinophil response, which in turn has been shown to be modulated by T-cell mechanisms. Therefore, this study was performed to study the relationship between eosinophilia, granuloma formation, and host morbidity in athymic mice infected with S. mansoni.

Animals. Congenitally athymic Nu/Nu and litter-mate heterozygote Nu/+, backgrossed to the BALB/c line for six generations, were obtained from the U.S. N.I.H., USPHS. Animals were maintained in germ-free isolators or under conditions of laminar flow.

Schistosome maintenance and animal exposure. The Puerto Rican strain of S. mansoni was maintained as previously described. Life-cycle support hosts were albino strain Biomphalaria glabrata snails and Walter Reed strain albino (WRm: (ICR) BR), or C57BL/6, mice. Cercariae were shed from infected snails by exposure to light, and mice were exposed by tail immersion and penetration by the cercariae within 90 minutes of photic release. Cercariae viability was judged by motility, and penetration was judged by counting residual head forms following exposure. Both criteria were met consistently with a frequency of greater than 95%.

Assessment of parasitic burden. Animals were killed by i.p. injection of 1000 milligrams sodium barbitol and 500 units of sodium heparin per kilogram of body weight. Mesenteric and hepatic vessels were perfused with 0.85 saline via aortic cannulation. The worms were collected on the filter paper disc using a suction apparatus and counted after staining with Lugol's iodine.

Thymic reconstitution. Thymic reconstitution (TxR) was accomplished via 4 newborn (0-36 hrs\_ thymus grafts, obtained from BALB/c mice, inserted subcutaneously beneath the axillary skin fold. Reconstitution was performed 6 weeks before exposure to cercariae and confirmed via the demonstration of a mitogenic response to Conconavalin-A in tissue culture by lymphocytes obtained from the reconstituted animals.

Determination of peripheral blood eosinophilia. Animals were bled between 1100 and 1300 hours from the lateral tail vein. Free-flowing blood was aspirated into white cell pipettes and diluted with Discombe's solution (5 parts 1% eosin, 5 parts acetone, and 90 parts water). The cells were counted in a Speirs-Levy chamber.

Histologic evaluation of granuloma formation. Tissue fragments were fixed in 10% neutral formaldehyde.  $5\mu$  sections were stained with Giemsa or hematoxylin-eosin. Granuloma size was determined by the measurement of two perpendicular diameters at the mid-transmiracidial level of the egg, utilizing a filar micrometer eyepiece (A.H. Thomas, Inc., Phila., Pa.). All sections were evaluated in a coded manner.

Determination of antibody formation. The Cercarienhullen reaction (CHR) was studied in a group of 6 Nu/Nu mice that were exposed to 150 cercariae. Six, 7 and 8 weeks after infection, 2 mice were deeply anesthetized by ether and were exsanguinated by heart puncture. Serum was harvested by conventional techniques and frozen. Sera were tested by placing two drops of each sample in a depression slide and adding 10 freshly shed cercariae to each preparation. The cavity was ringed with vaseline and sealed with a coverslip. Incubation proceeded for 2 hours at room temperature after which the parasites were examined under a microscope at 100x. Controls employed serum from Nu/+ mice infected with 150 cercariae 8 weeks earlier. CHR presence was graded by sheath size and expressed by +'s; ++++ represented a fully formed,  $7\mu$  strong CHR sheath, while 0 was used as the point indicating no distinct precipitate.

Specimens of duodenum and spleen were also obtained and processed utilizing a fluorescent antibody technique for the detection of tissue immunoglobulins. Specific antisera to mouse IgA, IgM, and IgG were employed.

Determination of portal pressure. Portal pressures were measured with a pressure transducer utilizing a solution of saline, containing 1% Tween-20 and 20 units of heparin per ml. Measurements were calibrated against the saline column and corrected appropriately. The needle was placed in the portal vein and pressures determined from above and below, one minute after entering the vein.

TABLE VII

Development of S. mansoni in Nu/Nu mice

		reception of D. menson in italian mine	וני נוו ואמיואמ ווונר		
No. of Cercariae	Animal	No. of Worms	% R.	WTM <sup>d</sup>	WTF
25	Nu/Nu	12.4 ± 1.4	49	0.35 ± 0.13	0.26 ± 0.06
25	Nu/+	$7.2 \pm 2.1$	29	$0.30 \pm 0.14$	$0.21 \pm 0.05$
100	Nn/Nn	$49.3 \pm 8.9$	49	$0.36 \pm 0.09$	$0.13 \pm 0.04$
100	Nu/+	$41.8 \pm 3.1$	41.8	$0.36 \pm 0.11$	$0.11 \pm 0.06$

<sup>a</sup> Number of exposure cercariae.

<sup>b</sup> Number of worms recovered by perfusion at 6 wk. <sup>c</sup> Percentage of recovery of worms (number of worms recovered/number of exposure cercariae)  $\times$  100. <sup>d</sup> Weight of male worms in milligrams: mean  $\pm$  95% confidence interval. <sup>e</sup> Weight of female worms in milligrams: mean  $\pm$  95% confidence interval, n  $\ge$  8.

TABLE X

Anatomic and functional morbidity in Nu/Nu mice after exposure to S. mansoni cercariae

Animal	Cercariae Exposure	Spleen Weight	Liver Weight	Portal Pressure	RESA
			0		
		Bw	000	cm/saline	×
Nu/Nu	0	$171 \pm 32$	$6.35 \pm 0.76$	$8.6 \pm 0.6$	$0.029 \pm 0.011$
	+	$347 \pm 108$	$7.18 \pm 0.62$	$10.9 \pm 1.8$	$0.038 \pm 0.009$
Nu/+	0	$130 \pm 39$	$5.32 \pm 0.21$	$6.2 \pm 0.5$	$0.016 \pm 0.007$
	+	$658 \pm 124$	$8.64 \pm 1.86$	$11.1 \pm 2.3$	$0.047 \pm 0.019$
Nu/Nu T×R	0	$162 \pm 51$	$5.77 \pm 0.33$	$7.4 \pm 0.6$	$0.024 \pm 0.003$
	+	$589 \pm 186$	$8.12 \pm 1.16$	$11.6 \pm 3.0$	$0.041 \pm 0.012$

Animals were exposed to 100 cercariae 9 weeks before study.
 Reticuloendothelial system activity: carbon clearance:

Determination of reticuloendothelial function. Reticuloendothelial activity was determined by the injection of a stable colloidal carbon suspension, 16 mg/gm body weight, as described by Corson. Freshly centrifuged Pelikan (Cll/1431a) was injected into the tail vein and repeated blood samples were removed from the retroorbital plexus, 1, 2, 4, 8, and 16 minutes subsequent to injection, utilizing heparinized calibrated capillary tubes. Following lysis in o.1% Na<sub>2</sub>CO<sub>3</sub>, carbon levels were determined by measurement of residual spectrophotometric light scatter. The rate of carbon clearance was calculated by the formula:

$$K = \frac{(\log conc. a - \log conc. b)}{(ta - tb)}$$

utilizing linear analysis of the curve of best fit, as determined by minimum "r" value.

<u>Data analyses</u>. All data given represent the mean + 95% confidence interval for that group. Statistical significance was evaluated using the Student's T test and linear transformations.

Assessment of parasitic burden. Nu/Nu/ and Nu/+ mice were exposed to 25, 50, or 100 cercariae and sacrificed 6 weeks later. As shown in Table VII, when animals were exposed to 25 cercariae, a slightly higher percentage of cercariae developed into adult worms in the Nu/Nu mouse when compared to the Nu/+ control. However, it must be added that this difference, while suggestive, was not significant at the 95% confidence level; no difference in survival was observed when 50 (data not shown) or 100 cercariae were utilized. The size of the worms which developed in the nude mouse were also slightly larger, although, again, this difference was not statistically significant.

Assessment of thymic reconstitution. As an index of thymic reconstitution, the spleen cells of selected animals were obtained at the time of cercarial exposure and cultured in the presence of Conconavalin-A (10  $\mu g/ml$ ). As shown in Table VIII, a significant increase in mitogen response was consistently observed. In addition, a significant increase in G<sub>1</sub> levels after thymic reconstitution was noted in 10 of 11 additional animals chosen at random for screening.

Determination of peripheral blood eosinophilia. It has been previously suggested that the development of eosinophilia following exposure to  $\underline{S}$ . mansoni may be the result of a mechanism dependent upon an adequate response by T dependent lymphocytes. To test this hypothesis, Nu/Nu, Nu/Nu/ TxR, and Nu/+ were exposed to 50 cercariae and bled at weekly intervals for the determination of blood eosinophil levels. As shown in Figure 40, the Nu/Nu mouse, when exposed to  $\underline{S}$ . mansoni, failed to develop significant eosinophilia when compared to either the Nu/+ or Nu/Nu TxR infected controls.

TABLE VIII

Effect of thymic reconstitution on response of Nu/Nu mouse spleen cells to mitogens

Animal	Addition of Con A	cmp <sup>a</sup>	N+/NT
Nu/Nu	+	$2.1 \pm 1.2$	0/14
	_	$1.1 \pm 0.7$	
Nu/+	+	$74.6 \pm 13.5$	12/12
	<u> </u>	$1.3 \pm 0.6$	
Nu/Nu T×R	+	$49.6 \pm 8.3$	11/12
	_	$1.4 \pm 0.9$	

 $<sup>^</sup>a$  Cpm 3H-thymidine incorporation. 0.5  $\times$  10  $^6$  cells were cultured in microtiter plates for 60 hr before a terminal 4-hr incubation in the presence of 0.5  $\mu$ Ci/ml of 3H-thymidine, preceding culture termination by an automated cell harvestor. Mean  $\pm$  95% confidence interval.

Determination of granuloma formation. Because a dominant cell type in the granuloma associated with egg deposition within the liver has been described as an eosinophil, it next became of importance to determine the size of the granulomata and the nature of the cellular constituents composing them. As shown in Table IX, the granulomata were significantly smaller in Nu/Nu mice when compared to either Nu/+ or Nu/Nu TxR control animals. Representative examples of the granulomas from Nu/Nu and Nu/+ mice are shown in Figure 41. Histologic examinations showed that the major cause of this decrease appeared to be a relatively selective diminution in the number of eosinophils within the granuloma. Lymphocytes and other mononuclear cells also appeared to be decreased, but to a lesser degree. However, granuloma disruption and exact cell enumerations were not performed. Granulomas from Nu/Nu TxR mice were indistinguishable from those found in Nu/+ animals both size and histologic composition. Adult worms were also occasionally observed in the livers of these mice. Again, it was noteworthy that virtually no reaction in Nu/Nu mice occurred about these worms (Figure 42). In contrast, infiltrates, consisting of a variety of mononuclear and polymorphonuclear cell types, were observed around the adult worms found in the livers of Nu/+ mice. Nu/Nu TxR mice exhibited a similar reaction to that shown by the Nu/+ animals.

Determination of antibody formation. In addition to a reduction of granuloma formation in Nu/Nu animals, there was also a significant impairment of the production of CHR antibody in this group. No reaction was detected in any serum from Nu/Nu mice, whereas positive CHR reactivity ranging from + to ++++ was seen utilizing sera obtained from all Nu/+ mice tested. Immunocytofluorescence studies demonstrated cells demonstrated cells producing immunoglobulins of all classes in the lamina propria of the small intestine and in the spleens in both Nu/Nu and Nu/+ mice. Quantitative comparisons were not attempted.

<sup>&</sup>lt;sup>b</sup> Number of animals showing a significant blastogenic response to Con A/number of animals tested.

DEVELOPMENT OF PERIPHERAL BLOOD EOSINOPHILIA IN NUDE MICE FOLLOWING EXPOSURE TO S. MANSONI CERCARIAE

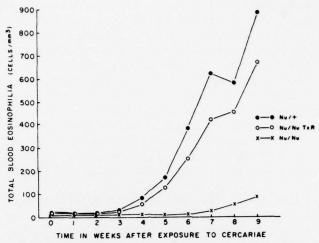


Figure 40 Development of peripheral blood eosinophilia in nude mice after exposure to S. mansoni cercariae. Nu/+ (•—••), Nu/Nu (×——×), and Nu/Nu TxR (○——○) mice were exposed to 50 cercariae and bled at weekly intervals for the determination of peripheral blood eosinophilia. Significantly greater eosinophilia was observed with the Nu/+ and Nu/Nu TxR animals when compared to the Nu/Nu animals.

Determination of host morbidity. It is postulated that the degree of host morbidity is in some way related to the granulomatous processes within the liver. Accordingly, an attempt was made to study various anatomic and functional characteristics of animals infected with 100 cercariae 8 weeks before sacrifice (Table X). Because of considerable variation in the "normal values" for each experimental group, comparisons of absolute values between all groups is difficult. However, comparisons between infected and uninfected members of each group is more feasible. All of the changes between infected and unexposed animals of a given group, as shown in Table X, are significant at the 95% confidence level with the exception of liver weight and reticuloendothelial clearance rate changes in the Nu/Nu group. Nu/Nu mice, when compared to Nu/+ or Nu/Nu TxR controls, developed considerably less splenomegaly and hepatomegaly. In addition, the portal pressure and reticuloendothelial clearance rates increased much less in Nu/Nu than the Nu/+ or the Nu/Nu TxR control animals. The relative changes in each of these parameters is summarized in Figure 42. Note that the percentage changes are plotted logarithmically.

These studies suggest that the development of the eosinophilia, granuloma formation, and host morbidity which is associated with infection by <u>S. mansoni</u> are controlled by thymic dependent immune mechanisms. In

this regard, they are consistent with previous observation in this area. Since thymic derived lymphocytes have recently been implicated in the production of a soluble mediator which is capable of stimulating the migratory characteristics of these cells in vitro and the level of circulating eosinophils in vivo, it is intriguing to implicate this as a possible mechanism in the mobilization and recruitment of cells for the formation of granulomas. Indeed recent studies have supported the hypothesis that the induction of blood eosinophilia, utilizing large latex particles coated with human gammaglobulin, may indeed also be mediated by T lymphocytes. Since these latter studies and schistosomiasis share the phenomenon of embolic delivery of large, relatively bound immunogens to the pulmonary capillary bed, it is intriguing to further conjecture that the eosinophilic response is in some way related to this unique anatomic compartmentalization of immunogen presentation.

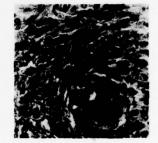
Previous studies by Colley, utilizing thymectomized-irradiated or thymectomized-anti-thymocyte serum treated mice, indicated that the histopathologic response to egg deposition in these animals is predominantly one of liquefactive necrosis with bacterial invasion and severe parenchymatous destruction. Although the granulomas formed in these





Nu/+ Heterozygote





Nu/Nu Homozygote

Figure 47 Histologic sections of representative granulomas in Nu/+ and Nu/Nu mice. Significantly larger, more cellular granulomas were noted with the Nu/+ animals, hematoxylin-eosin  $\times$  100. Higher magnification of granulomas demonstrates that a major cause for the decreased cellularity of Nu/Nu mice of the granulomas was due to the relatively selective depletion of eosinophils, ( $\leftarrow$ ), hematoxylin-eosin  $\times$  600. (Granulomas found in Nu/Nu TxR animals were indistinguishable either by size or histologic composition from those found in Nu/+ animals.)

animals were small, the resultant clinical course of the infected animals was one of marked morbidity and mortality. In contradistinction, the nude mouse apparently develops suppressed granuloma formation and actually exhibits reduced morbidity when assessed in terms of organ size, portal hypertension and reticuloendothelial activity. These latter two functions may be general physiologic correlates to the severity of the development of pathologic reaction. It can be conjectured that the Nu/Nu mouse retains certain mechanisms relating to its ability to deal with the invading organisms, which might be lost by the exogenously suppressed animal. In addition, these Nu/Nu animals were maintained in germ-free isolators and perhaps protected from infection or other environmental stresses with greater efficiency.

The observation that increased eosinophilia, granuloma formation, organ size, portal pressures, and reticuloendothelial activity occurred in the thymic reconstituted animals clearly showed that the increase in morbidity, due to the infection, was a result of interactions involving thymic dependent immune mechanisms. Indeed the Nu/Nu TxR animals more closely resembled the Nu/+ than the Nu/Nu animal. This is taken as strong evidence that the thymic function of the animal was responsible for the observed differences between the Nu/+ and Nu/Nu animals. Decreased morbidity in congenitally deficient mice when compared to phenotypically normal litter mates is not unique to schistosomiasis. For example, increased resistance of Nu/Nu mice toward African trypanosomiasis and systemic candidiasis has been observed.

TABLE IX

Granuloma formation in Nu/Nu mice

Animal <sup>a</sup>	Granuloma <sup>b</sup>	Percentage	Probability
10.00	μm		
Nu/+	$197 \pm 38$		
Nu/Nu	$76 \pm 21$	38	< 0.05
Nu/Nu T×R	$162 \pm 41$	82	NS'

 $<sup>^</sup>a$  Animals were exposed to 100 S. mansoni cercariae 9 weeks before sacrifice.

- b Mean granuloma diameter ± 95% confidence interval.
- Percentage of heterozygous control granuloma size.
- <sup>d</sup> Statistical significance vs Nu/+ control; Student's t-test.
- NS, not significant.

These studies represent a preliminary description of the response of Nu/Nu mice to infection with Schistosomiasis mansoni. Although they clearly indicate a thymic dependency of the eosinophilia, granulomatous hypersensitivity, and host morbidity upon intact thymic function, a number of questions remain unanswered. For example, what are the alterations of effector mechanisms whereby the basic pathophysiology is altered within the Nu/Nu mouse? Studies employing soluble egg antigen induced blastogenesis, immunogen binding, antibody formation, mediator

release and subpopulation analysis will be necessary to answer this question.

The failture to observe antibody production as determined by the CHR test is an example of the complex relationships which must be evaluated. The findings are consistent with a T-cell helper influence upon B cell antibody production. Previous studies have emphasized an inverse relationship between T cell competence and antibody formation in normal mice. It is probable then that both the immune responses and the secondary or modulating responses will be altered in the nude mouse model. It may be of interest in this regard that it has not been possible to produce accelerated granuloma formation in the nude mouse.

# DEVELOPMENT OF ANATOMIC AND FUNCTIONAL MORBIDITY IN NUDE MICE FOLLOWING EXPOSURE TO S. MANSONI CERCARIAE

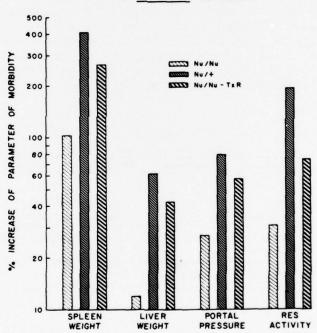


Figure 42 Development of anatomic and functional morbidity in mice after exposure to S. mansoni cercariae. Nu/Nu (\simple ), Nu/+ (\simple ), and Nu/Nu TxR (\simple ) animals were sacrificed 8 weeks after infection with 100 cercariae. A number of parameters were studied: spleen weight, liver weight, portal pressure, and reticuloendothelial activity. Significantly greater morbidity, judged by the above four criteria, was noted in the Nu/Nu animals (Note: Scale is logarithmic.)

Another area of concern involves the importance of T cell dependent mechanisms in host resistance to S. mansoni. Such mechanisms have been elucidated in the rat. However, mechanisms may well not be analogous between the two species. It would appear that gross worm development in nude mice is excellent, perhaps slightly superior to that which occurs in heterozygotes. However, a precise ultrastructural analysis and functional evaluation of the worms remains to be performed. Significant questions also remain to be asked regarding the importance of T cell mediated mechanisms in the resistance to reinfection and ability to modulate granuloma formation. Here it is of interest to note that although nude mice produce a variety of antibodies against schistosomal antigens, they develop only limited augmented resistance to reinfection, and their cells fail to manifest a variety of in vitro reactivities against schistosomal immunogens including blast transformation and the release of various biologically active products. Similarly, a decreased histologic response against intravenously injected schistosomula has been observed in previously exposed nude mice by Sher, et. al. It is hoped that subsequent studies, involving the use of nude mice may help to clarify some of these mechanisms of resistance to and morbidity from schistosomiasis.

#### 12. The immune response to schistosomiasis in rats.

Schistosomiasis represents a world health problem of immense proportion affecting an estimated 200-300 million individuals. Intensive efforts utilizing a variety of control techniques have indicated that current public health efforts per se are inadequate to prevent the increasing spread of the disease. Accordingly, control through immunoprophylaxsis and treatment through immunotherapy might be of immense benefit. A better understanding of those mechanisms which are operative in the immune response to schistosomiasis is a prerequisite for the optimal development of such a program, and intense effort has been devoted to the assessment of host resistance phenomenon which may occur either innately or "naturally" or be induced by specific immunologic mechanisms.

Recently, a variety of experimental models have been developed which provide the opportunity to more accurately assess host defense mechanisms during schistosome infection. We have studied previously the characteristics of the cellular and humoral response to S. mansoni infections in inbred rats during initial exposure. The results indicate that the kinetics of this infection were strongly influenced by the age of the host and the magnitude of cercariae exposure. The initial immune response to infection appeared to be mediated by a population of thymusderived lymphocytes. The ole of serum was complex: it appeared to modulate cellular immunit and to directly promote worm survival in a manner analogous to classic enhancement during the earlier phases of infection; subsequently, as the protective capacity of lymphocytes appeared to wane, antibody apparently developed a more dominantly protective role.

The clinical setting of schistosomiasis, however, involves repeated exposures. Accordingly, our studies were extended to evaluate the cellular and humoral responses to S. mansoni infections in inbred rates during re-exposure. These initial experiments describe the response of Fischer rats to re-exposure to S. mansoni and define the kinetics of the observed, immunologically mediated, augmented resistance. In addition, they define the nature of the target and describe certain basic aspects of the mechanisms of the phenomenon of immunologic resistance.

Schistosome maintenance and animal exposure. The Puerto Rican strain of S. mansoni was maintained as previously described. Life cycle support hosts were albino strain Biomphalaria glabrata snails and Walter Reed strain albino Wrm: (ICR)BR mice. Vertebrate exposures and assessment of parasite burdens were performed as described in previous experiments. Unless otherwise noted, 4-1/2 to 5-week-old (40-60 gm) male rats of the Charles River Fischer strain (CDF) were used in the study. A total of 2,640 animals were used.

Radioisotopic techniques. Radioactive populations of cercariae were produced by the addition of one  $\mu \text{C}i$  per ml of water of tritiated amino acids at the time of initial confirmation of intramolluscan infection. Fourteen to 21 days after the addition of the radioactivity, the snails were exposed to light and the resultant cercariae utilized as the radioactive population of infecting cercariae. The worm populations were obtained by perfusion, heat fixed and dipped in Kodak NTB-2 emulsion. Following 10-16 weeks of incubation under dessication at 4°C, the radioautographs were developed using Kodak Dektol solution. Radio-labelling was determ ned by microscopic examination under dark field illumination. Cercariae of schistosomules were considered to be positive if the number of grains above the work was three-fold that of the surrounding background density. The details of this methodology have previously been described.

<u>Cell-transfer experiments</u>. Peritoneal exudate cells were harvested by peritoneal lavage with iced L-15 media (Leibovitz, GIBCO), containing heparin (10 units per ml without preservative, Flow Laboratories), three days following an intraperitoneal injection of 25 ml of light mineral oil (Marcol 55). The cells were washed twice in media, then filtered at 37° through columns containing Fenwall nylon wool as previously described. The non-adherent population was washed and subsequently utilized in the cell transfer experiments.

Serum transfer experiments. Animals were exsanguinated by cardiac puncture. Blood was allowed to clot at  $37^{\circ}\text{C}$  for 30 minutes, and to retract for four hours at  $4^{\circ}\text{C}$  and was then centrifuged at 1500 kg for 20 minutes at  $4^{\circ}\text{C}$ . The sera were stored at  $-70^{\circ}\text{C}$  until used. Immediately before intravenous injection into recipient animals, all sera were again centrifuged and sterilized by filtration (0.45 m $\mu$ , Millipore Corp.). Sera were fractionated as previously described. Procedures included:

Gel filtration utilizing Sephadex G-200 (Pharmacia); QAE chromatography utilizing QAE-Sephadex A-50 (Pharmacia); and Immunoadsorbent chromatography utilizing goat anti-rat IgG, covalently linked to sieved G-200 Sephadex (Pharmacia) by cyanogen bromide.

In Vitro determination of antibody activity. Indirect hemagglutination antibody activity was determined, utilizing sheep red blood cells to which schistosomal antigen had been previously coupled through the use of tannic acid. Complement fixation activity was determined utilizing antigen block titrations and freshly standardized guinea pig serum.

Determinations of the development of resistance. Animals were exposed to an initial cercarial  $(C_{OWk})$  population and, 4 weeks later, divided into two groups. A portion of the animals served as an initial exposure control, and a portion was exposed to a second, radiolabeled population of cercaria  $(C_{LWk})$ . Additional age-matched animals were exposed only to the  $C_{LWk}$  infection. Specific augmented resistance to the second or re-exposure cercariae population was calculated by the formula:

$$\frac{A - B}{A}$$
 x 100,

where: A = the number of worms resulting from the  $C_{4wk}$  single exposure, and B = the number of worms surviving from the  $C_{4wk}$  population of doubly exposed animals.

When radiolabeled populations of worms were not used, augmented resistance was calculated by the formula:

$$\frac{A - (B-C)}{A} \times 100,$$

where: A = the number of worms recovered from challenge ( $C_{l_wk}$ ) infection control animals, B = number of worms recovered from doubly infected animals, and C = the number of worms recovered from the primary exposure ( $C_{Owk}$  control animals).

<u>Data analysis</u>. All data points represent the mean, and intervals represent the 95% confidence interval of group determinations  $(n \ge 6)$ . Statistical analyses utilized Student's t-tests and arithmetic conversions.

Kinetic studies on the development of augmented resistance following initial exposure to S. mansoni. Previous studies have indicated that following an initial exposure to S. mansoni, the rat developed augmented resistance to reinfection. To more accurately assess this phenomenon, it became necessary to discretely evaluate multiple populations of schistosomules developing simultaneously within the same host. It was possible to follow with great accuracy the distinct subpopulations of worms through the method of radiolabelling selected populations (Table XI).

TABLE XI

Effect of Initial Exposure to Cercariae upon Subsequent Exposure Development

Age of exposure <sup>a</sup>	Number of worms <sup>b</sup>	Number of worms positive <sup>c</sup>
4 —	18 ± 7	0
- 8 • d	$97 \pm 13$	95 ± 18 ●
4 8 ●	$24 \pm 11$	7 ± 6 ●

<sup>a</sup> Animals were exposed to 1000 S. mansoni cercariae at either 4 or 8 weeks of age, or at both 4 and 8 weeks of age.

<sup>b</sup> Mean number of worms recovered at perfusion.

· Mean number of worms bearing tritiated amino acid by radioautographic criteria.

d The cercariae population used to expose animals at 8 weeks of age was obtained from snails cultured in water containing tritiated amino acids (♠). Animals were also exposed at 8 weeks to unlabeled cercariae and simultaneously injected with tritiated amino acids (10× the cpm of that shown by radiolabeled cercariae). Worms, 14 of 460, which developed from that cercariae pool were subsequently found to carry significant amounts of tritiated amino acids by radio-autographic criteria.

Animals were exposed to 1000 cercariae each either at 4 weeks of age, at 8 weeks of age, or at both 4 and 8 weeks of age. The cercariae utilized for the 8 weeks exposure were labeled with tritiated amino acids. Utilizing radioautography, virtually all of the radioactive worms could be detected via the demonstration of their retention of significant radioactivity. In addition, when animals were doubly exposed, the second population of cercariae developed very poorly (here shown as a reduction from a mean of 95 to 7 worms, or approximately 90%). it is also of interest that the number of worms, surviving from the initial infection, was not significantly affected by the exposure to a second cercarial population. That is, the mean (24-7) of 17 unlabelled worms present after double exposure in the third group is very similar to the mean of 18 worms seen in the (single exposure) first group.

Initial studies also demonstrated that the pattern of resistance development was critically dependent on a number of factors. One of these was the number of cercaria which constituted the initial exposure. In studies employing varying numbers of cercariae in the initial exposure, it was found that augmented resistance to reinfection could be induced by exposure to as few as 30 worms (Figure 43). Resistance continued to increase, with increasing initial exposure, reaching a maximum following an initial exposure of between 300 and 1000 cercariae. Because of these findings, all subsequent studies utilized an initial exposure of 1000 cercariae.

Our studies next turned to the rate of development of specific resistance to re-exposure to  $\underline{S}$ .  $\underline{\text{mansoni}}$ . Animals were exposed to an initial infection of  $\underline{S}$ .  $\underline{\text{mansoni}}$  and then reexposed simultaneoulsy and/or at weekly intervals thereafter to a second, radiolabeled population of worms. Specific augmented resistance, again defined as a significant reduction in the number of developing adult worms from the second exposure, occurred as early as one week following initial exposure to  $\underline{S}$ .  $\underline{\text{mansoni}}$ . A peak of resistance was reached approximately 6 to 8 weeks after exposure (Figure 44).

The effects of the initial exposure upon the development of the second population of cercariae into adult worms was apparently reflected both in terms of quantitative recovery of worms and qualitative effects upon them (Figure 45). Animals were initially exposed to 1000 unlabeled S. mansoni cercariae. They were subsequently exposed either 7 or 21 days later to radiolabeled cercariae. Simultaneously previously unexposed, age-matched control animals were exposed for the first time to radiolabeled cercariae. The animals were then sacrificed 21 days later and perfused. The isotope retention per form was determ ned and compared to the radioactivity incorporated into the initial schistosomule preparation. The results indicate that with increasing resistance, indicated here as the dotted line representing the per cent reduction of challenge worm burden, the percentage of isotope retained by the worms also increased. These data are consistent with an adverse quantitative effect upon the general metabolic activity of the worm. With this decrease of general metabolic activity, a decrease in the turn-over rate of the endogenous radiolabeled aminoacid pool and an increase in the percentage of retained radiolabel would be predicted. The results are consistent with such an interpretation.

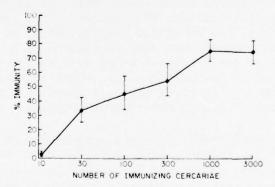


Fig. 43 Relationship between number of immunizing cercariae and the subsequent development of immunogically mediated resistance: Animals were exposed to increasing numbers of cercariae and challenged 6 weeks later with a second population of radiolabeled cercariae. The specific reduction in survival of the second population is shown as the percentage immunity. Significant protection was noted following exposure to as few as 30 cercariae. The development of maximum resistance required an initial exposure of approximately 1000 cercariae.

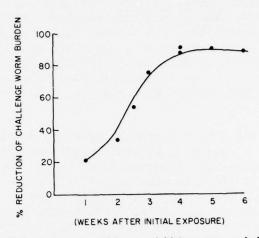


FIG 44 Relationship between the interval between initial exposure and challenge and the development of immunologically mediated resistance: Animals were exposed to 1000 cercariae and challenged at weekly intervals thereafter with 3000 additional radiolabeled cercariae. The specific reduction in survival of that second population is shown as the reduction of challenge worm burden. Reductions in survival of the second population were observed within 1 week of initial exposure. Maximum resistance was noted 5-6 weeks following initial exposure.

Additional evidence for a qualitative effect of previous host immunzation upon subsequent worm development was obtained from morphologic analyses. When normal animals were perfused 18 days after initial exposure to S. mansoni cercariae, 54 per cent of the recovered worms were sufficiently developed to enable classification by sex. In comparison, when previously exposed rats were re-exposed to S. mansoni, the sex of only 31 per cent of the recovered worms from the re-exposure population could be determined morphologically. These findings are compatible with an adverse effect of previous exposure upon the growth and morphologic differentiation of subsequent exposure populations.

Studies on the mechanisms of specific augmented resistance which results from an initial exposure to S. mansoni. The initial studies indicated that augmented resistance in the rat could occur very rapidly after initial exposure and, therefore, implied that immunity could be efficiently stimulated by very early stages of infection. Subsequently, the immune response appeared to be directed against an early stage of infection.

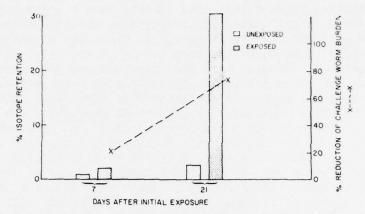


Fig. 45 Relationship between the development of immunologically mediated resistance as assessed by worm survival and the quantitative retention of radiolabel by the surviving worms: Animals were exposed to 1000 unlabeled cercariae. Seven and twenty-one days later they were reexposed to a second population of radiolabled cercariae ( ). Simultaneously, age-matched control animals were exposed for the first time to the same cercarial pool (). The percentage isotope retention per surviving worm was determined by the formula (cpm/worm ÷ cpm/cercariae) × 100. A significant increase in the percentage of retained \*H-labeled amino acids was observed by the worms which were obtained from animals that demonstrated strong resistance to the second injection, as determined by a reduction in survival of the population (×---×).

To further assess this apparent stage specificity, the survival of each population of worms was determined in experiments utilizing an exposure of animals to: an initial or primary control population of cercariae; a secondary or challenge control population of cercariae; or to both populations of cercariae. Exposure to a primary infection was followed at serial intervals by exposure to a challenge population. The simultaneous exposure to cercariae, in the numbers utilized, had no significant effect on the survival of the opposite population (Figure 46). However, within one week there was significant augmented resistance demonstrated against the second or challenge population. The survival of both primary and challenge (reexposure) worm populations are shown for all three rat populations. No significant effect on the survival of the initial population as a result of exposure to the second population was observed (Ovso). This specific resistance, also known as concommitant immunity, was subsequently directed with increasing efficiency against only the challenging population, shown as the difference between exposure and control worm recovery ( vs ). This stage specificity of resistance is summarized in Figure 47. Resistance against both populations of worms, derived from differential survival in animals simultaneously bearing both populations, is determined by comparison to survival of the appropriate control population in animals exposed to only that control population.

These initial studies indicated clearly that the development of resistance could be stimulated by early stage-specific immunogens. It therefore became important to ascertain if there was a similar stagerelated specificity shown by the worm target of the effector aspects of this resistance. Studies were next performed to determine the nature of the target of the immune system during reinfection. Nonadherent peritoneal lymphocyte populations or serum were obtained from animals who had been previously exposed to S. mansoni, 2-1/2 or 7 weeks respectively. The cells or serum were injected i.v. into syngeneic recipients at various time intervals following the exposure of these animals to cercariae. Cells were effective at transferring adoptive immunity only if they were introduced within the first 72 hours following exposure to cercariae (Figure 48). When an interval exceeding 72 hours was utilized, cells showed no demonstrable effect on the development of the cercariae into adult worms. A nearly identical early stage specificity was shown with the transfer of protective serum. As shown in Figure 49, sera were effective only if transferred within % hours of initial exposure to cercariae. These studies indicate that the specific augmented resistance is apparently directed against very early stages of schistosomule development.

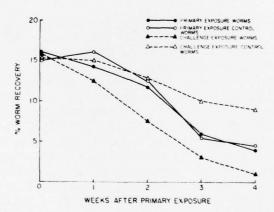


Fig 46 Simultaneous survival of subpopulations of worms within the same animal. Animals were exposed to an initial or primary exposure of 1000 cercariae ( $\bigcirc$ ); both to an initial ( $\bullet$ ) exposure of 1000 cercariae and to a challenge exposure ( $\triangle$ ) of 3000 radiolabeled cercariae 4 weeks later; or to only the 3000 radiolabeled cercariae ( $\triangle$ ). The survival of all four worm populations is shown as a function of the time interval between primary and challenge exposure. Specific resistance against the challenge population ( $\triangle$ — $\triangle$ ) was observed within 1 week of initial exposure. No significant effect on the survival of the primary exposure ( $\bullet$ ) resulting from exposure to the challenge population ( $\bigcirc$ — $\bullet$ ) was observed.

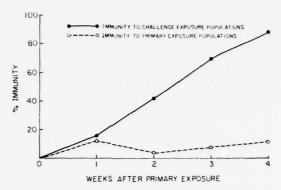


Fig. 47 Sequential development of stage-specific resistance to reexposure. Animals were exposed as described for Fig. 4. The specific effect of an initial exposure to cercariae to stimulate resistance against subsequent exposure (•) is compared to the effect of subsequent exposure upon the survival of the primary or initial exposure population (○). The percentage immunity is expressed as the specific reduction in survival of the given worm population in the doubly exposed animal when compared to the survival of the analogous population in the appropriate singly exposed animal. Specific resistance directed exclusively against the challenge population is observed within 1 week following initial exposure.

Studies on the immunologic nature of the specific resistance phenomena. Our previous studies had implicated the immune system as responsible for resistance in the rat to initial infection by S. mansoni. The importance of the immune system in mediating resistance during reinfection was, accordingly, next investigated.

Studies were first performed to demonstrate the general anamnestic characteristics of the rat's response to re-exposure to S. mansoni. Re-exposure of rats to S. mansoni led to a rapid augmented antibody response as judged by indirect hemagglutination or complement fixation (Table XII). In addition, the relative ability of a given volume of serum to induce protection, when adoptively transferred to syngeneic recipients, was similarly increased following a second exposure to S. mansoni. These data indicated that the serological response of rats to S. mansoni, during re-exposure, appeared to involve the previous generation of a population of memory cells and a subsequent anamnestic or secondary response upon re-exposure to cercariae.

Finally, studies were performed using the transfer of cells or serum in an attempt to ascertain the nature of the mediation of this augmented resistance. Previous experiments had determined the ability of cells or serum, obtained from previously exposed animals, to adoptively transfer resistance. Figure 50 summarizes the pattern of resistance as a function of time, following initial exposure. These pre-established

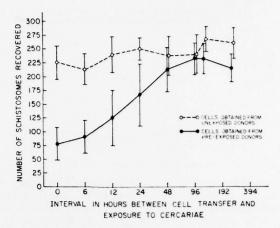


Fig. 48 Relationship of the interval between exposure to cercariae and transfer of cells and the subsequent demonstration of adoptive transfer of resistance: Animals were exposed to 1000 cercariae; 2.5 weeks later they were injected with 30 ml of light mineral oil. Nonadherent peritoneal exudate cells, 10°, were injected iv into syngeneic recipients at serial intervals following the exposure of these recipients to 1000 S. mansoni cercariae. Significant transfer of adoptive immunity was observed only if the interval between exposure and transfer did not exceed 36 hr.

patterns were used to design a series of experiments which varied the intervals between initial and subsequent exposures in an attempt to elucidate the mechanisms of immunity which were operative during re-exposure. When serum was obtained from animals which were exposed either 14 weeks or 3 weeks before sacrifice, the sera failed to demonstrate any significant protective ability (Table XIII). However, when serum was obtained from animals exposed both 14 and 3 weeks prior to sacrifice, the sera demonstrated strong protection upon adoptive transfer. Sera obtained from animals exposed 17 weeks before sacrifice similarly showed no significant protection.

Additional studies (Table XIV) have indicated that the protective moiety in the serum resided in the Ig fraction on QAE chromatography and 7S fraction on G-200 chromatography. Furthermore, protective activity could be removed utilizing a goat anti-rat immunoglobulin serum, covalently bound to Sephadex by cyanogen bromide.

Similar studies were performed utilizing the transfer of unfractionated spleen cells. Table XV illustrates one such study. Cell populations obtained from animals, exposed 3 weeks previously to  $\underline{S}$ .  $\underline{mansoni}$ ,

demonstrated significant protection. However, cell populations, obtained from animals exposed 10 weeks before sacrifice or both 10 weeks and again 3 weeks before sacrifice, both failed to demonstrate any significant protection. A total of ten similar experiments utilizing a variety of timing intervals between initial and subsequent exposure and sacrifice also failed to show any evidence for the transfer of resistance by cells after re-exposure.

These data suggest that the secondary resistance response to  $\underline{S}$ .  $\underline{\text{mansoni}}$  is at least in part mediated through the development of high levels of protecting antibody. However, direct cellular mechanisms were not demonstrated.

These studies clearly indicate that the rat develops strong and effective immunity within one week following initial exposure to  $\underline{S}$ .  $\underline{\text{mansoni}}$ . Moreover, the general characteristics of this response  $\overline{\text{appear}}$  to be analogous to the anamnestic or secondary immune response. The rapidity with which this immunity develops suggests that it may be stimulated by immunogens which are present on the very early forms of schistosomules. Hence, the stimulation of augmented resistance is essentially complete within the rat before adult forms or egg antigens are present within the animal.

The resistance which develops as a result of stimulation of the rat immune system by very immature forms is apparently also specifically directed against those same immature forms upon re-exposure. This phenomenon was demonstrated by the rapid development of protective immunity against the immature worms encountered during re-exposure with little or no deleterious effect on those worms had survived from the initial infection. This phenomenon, also known as concommitant immunity, has been previously described by a number of investigators.

Transfer experiments indicated that cells and sera, to be effective in producing immunity, must be present within two to three days following exposure to cercariae. Schistosomules which have gone through 3 days of development in vitro have also been shown to be refractory to the actions of cells and sera which are added after this time period.

The explanation of this stage specificity is not known. Several possibilities exist, including: (1) the expression of unique stage-specific immunogen determinates of relevance to the protective immune response; (2) a non-specific quantitative susceptibility of immature forms to attack by adverse factors, perhaps related to the trauma of penetration; (3) or tissue-specific host factors, perhaps related to the anatomic distributional patterns of the migrating schistosomule.

The findings that the immune system in the rat can apparently reflect its activity both in qualitative and quantitative manner upon cercarial development into schistosomules is of additional interest. Recent studies by Knopf have indicated that, following the marked

spontaneous decrease in worms recoverable by perfusion and in association re-exposure, increased numbers of worms can be recovered in the liver. There is apparently minimal host reaction about these worms, and they are slowly destroyed. One possible interpretation of these findings is that those qualitative changes in the functional capability of the worm, which are induced by the immune response, prevent the worms from successfully holding residence in the peripheral splanchnic beds. Their inability to resist the pressures of blood flow would result in the subsequent hepatic shift.

The strength of the immunity which develops as the result of initial exposure to <u>S. mansoni</u> is related to the magnitude of the initial exposure. However, extensive studies are in progress to ascertain the

TABLE XII

Detection of Antibody Activity against S. mansoni in Sera of Rats

Previously Exposed to Cercaria

Exposure	regimen		Assay	
D-45ª	D156	C.F.¢	1.H.A.4	W.B. (%)
0	0			+ 6
+	0	8	160	-41
Ó	+	2	8	- 5
+	+	64	1028	-68

- Animals exposed to 1000 S. mansoni cercariae 45 days before exsanguination.
- <sup>b</sup> Animals exposed to 1000 S. mansoni cercariae 15 days before exsanguination.
- \* Complement fixation titer: reciprocal of the highest positive dilution.
- Indirect hemagglutination titer: reciprocal of the highest positive dilution.
- Percentage reduction of worm burden: 3 ml of sera were injected iv 2 hr before exposure of recipient animal to cercariae.

exact relationships between the absolute number of infecting cercariae, and the temporal patterns with which the cercariae are introduced into the rat, upon the resultant immunity. Attention will be paid to the optimal development as well as to the longevity of the development of the immunity which results. Preliminary studies have indicated that multiple small exposures are far more effective at inducing lasting immunity than a single exposure, even if that single exposure is of much greater magnitude than the total of the multiple small exposures. In addition, there is some evidence that very large exposures to S. mansoni may actually result in the development of suboptimal immunity. The significance of this phenomenon is at present not clear. However, the recent studies of Waksman and Feldmann indicate that very large doses of

immunogens may apparently result in either the production of inhibitors of DNA synthesis and, therefore, of effector cells, or bypassing macrophage dependent systems leading to the development of a form of functional tolerance. Both of these possible mechanisms are currently under consideration and active investigation.

Although the mechanism of this specific augmented resistance is not clear, it obviously involves in part the production of protective serum antibody. Moreover, antibody which demonstrates the ability to protect occurs within three weeks of secondary exposure. As previously observed, it took seven to ten weeks after initial exposure for serum antibody titers to be produced which demonstrated significant in vivo protection. The failure of cells to demonstrate protection following a second exposure must be interpreted with extreme caution. Preliminary studies from our laboratory have indicated that the cells obtained from animals which are multiply exposed to S. mansoni represent an extremely heterogeneous population. Although the unfractionated cell populations do not demonstrate significant protection, there is evidence that specific subpopulations within this general heterogeneous population may possess a protective capability. Their ability to demonstrate this protective capability, however, is modulated by the presence of other

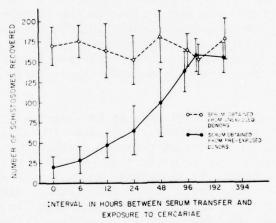


Fig. 49 Relationship of the interval between exposure to cercariae and transfer of serum and the subsequent demonstration of adoptive transfer of resistance: Animals were exposed to 1000 cercariae; 7 weeks later they were exsanguinated. Four milliliters of sera were injected iv into syngeneic recipients at serial intervals following the exposure of these recipients to 1000 S. mansoni cercariae. Significant transfer of adoptive immunity was observed only if the interval between exposure and transfer did not exceed 72 hr.

TABLE XIII

Effect of Exposure Pattern upon Subsequent Transfer of Immunity by Serum

Exposur interval	Number of worms <sup>b</sup>	Pe
	168 ± 25	-
14 —	$159 \pm 22$	>0.05
- 3	$171 \pm 31$	>0.05
14 3	$36 \pm 7$	< 0.001

a Interval between initial exposure to cercariae and subsequent sacrifice of animal to obtain sera subsequently used for adoptive transfer of resistance.

<sup>6</sup> Number of worms recovered from syngeneic animals which were injected iv with 2.0 ml of serum concomitant with exposure to cercariae and perfused 21 days later.

<sup>6</sup> P value vs number of worms obtained from animals injected with serum obtained from animals not previously exposed to S. mansoni.

d Animals exposed to S. mansoni 14 weeks and again 3 weeks prior to sacrifice.

cell populations. For example, recent studies have indicated that circulating immune complexes may be potent suppressors of the immune response either directly or through the stimulation of the development of thymic derived suppressor populations or the production of anti-receptor antibody. We have demonstrated such complexes in the serum of rats, immediately preceding the ostensible loss of the ability to adoptively transfer resistance by cells obtained from these animals. The nature of these specific cell populations is currently under intense investigation.

Finally, an additional caution must be exercised in interpreting these results. The rat is extremely resistant to initial infection by S. mansoni, demonstrates a dramatic immunologically mediated decrease in parasitic burden shortly after initial exposure, and virtually no morbidity from infection. It represents an animal in which host defense mechanisms, be they immune or non-immune, are highly effective, and, therefore, analogies between the rat and more sensitive hosts, such as man or mouse, must be drawn with caution. Alternatively, however, the rat may represent the ideal model to study the various salient defense mechanisms which are operative during both the initial and subsequent re-exposure to S. mansoni. However, analogy to human S. mansoni infection or to other experimental models such as the mouse, and the biological relevance of these observations remain to be elucidated.

# 13. The activity of a chloroindazole analog of hycanthone (IA-4 Noxide) against Schistosoma mansoni infection in rhesus monkeys.

In recent years, the antischistosomal drug, hycanthone, has come under increasing criticism as a result of its reported side-effects. Hycanthone produces mutagenic activity in a variety of submammalian and mammalian systems, both in vitro and in vivo. It has a direct action on spermatogenesis in the rat and produces semisterility. Teratogenicity has been demonstrated in mice and in rabbits. It induces malignant transformations in Rauscher virus-infected rat embryo cells and hepatocellular carcinomas in schistosome-infected mice. Structural modification of the hycanthone molecule has resulted in the synthesis of several drugs having greatly reduced mutagenic activity and decreased toxicity for mice, yet retention of the excellent antischistosomal property. One of these compounds, 8-chloro-5-hydroxy-

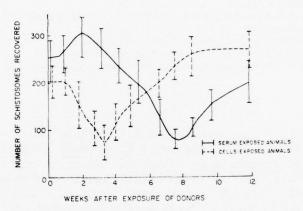


Fig. 50 Relationship between duration of infection and the adoptive transfer of resistance to S. mansoni by cells and serum. Animals were exposed to 1000 cercariae. At weekly intervals they were sacrificed and the serum and nonadherent peritoneal exudate cells were obtained. Three milliliters of serum or  $2 \times 10^8$  cells were injected into syngeneic recipients 4 hr before exposure to 1000 S. mansoni cercariae. Significant adoptive transfer of resistance was observed when cells were obtained from animals that had been previously infected approximately 2-3 weeks before sacrifice. The transfer of resistance was also observed when serum was obtained from animals that previously had been infected approximately 7-10 weeks before sacrifice.

methylbenzothiopyranoindazole N-oxide, designated IA-4 N-oxide (Figures 57), has a chemotherapeutic index in schistosome infected mice more than 12 times higher than that of hycanthone and only 1.5% of hycanthone's mutagenic activity in Salmonella. Chemotherapeutic index is established as the ratio of the median single intramuscular lethal dose (LD<sub>50</sub>) to the single intramuscular dose producing 50% reduction in the number of schistosomes (ED<sub>50</sub>). This report describes the antischistosomal activity of IA-4 N-oxide in rhesus monkeys.

TABLE XIV

Effect of Serum Fractionation upon Passive Transfer of Protection

Serum preparation	Number of worms	$\Delta^f$	$P^{g}$
Exposed			
Whole <sup>a</sup>	$43 \pm 21$	-146	< 0.01
Ig (QAE)b	$69 \pm 28$	-120	< 0.01
7S (G-200)°	$54 \pm 19$	-135	< 0.01
$-\gamma G (GARIgG)^d$	$162 \pm 37$	- 27	>0.05
Unexposed*	$189 \pm 48$	_	_

 $^{a}$  Whole serum (2.0 ml) obtained from animals which were exposed to S. mansoni cercariae 14 and 3 weeks before sacrifice.

<sup>b</sup> Immunoglobulin containing fraction obtained for QAE-Sephadex fractionation, 2 ml equivalent (v: v).

• 7S fraction obtained from G-200 column chromatography; 4S + 19S fractions did not demonstrate a significant protective effect.

 $^d\gamma G$  globulin depleted fraction obtained by affinity column chromatography using column-bound goat anti-rat  $\gamma G$  antibody.

\* Serum obtained from animals unexposed to S. mansoni cercariae.

f Decrease in number of recovered worms from unexposed animal (e).

• P value vs animals injected with serum from unexposed animal (Student's t test).

Ten young adult rhesus monkeys (Macaca mulatta) were experimentally infected with the Puerto Rican strain of S. mansoni maintained in our laboratory in albino Biomphalaria glabrata. Prior to exposure, routine stool examinations were performed to detect possible natural parasite infections. All animals weighed between 3.4 and 5.5 kilograms and appeared to be in good health. Percutaneous exposures were accomplished by applying approximately  $400 \pm 50$  cercariae to the clipped abdomen of anesthetized monkeys. No chemical restraints were used subsequent to exposure. Throughout the experiment, monkeys were maintained in individual cages on standard monkey chow and water ad libitum, supplemented with fresh fruit twice weekly.

Treatments were instituted according to the schedule outlined in Table XVI. An aqueous solution methane-sulfonate salt of IA-4 N-oxide was administered according to the body weights of individual monkeys. Oral administration was by naso-gastric intubation while intramuscular administration was made into the posterior thigh. Therapeutic treatment was initiated (day 50) after confirmation by fecal egg excretion analysis of a well established infection. The limited amount of drug available did not permit the oral therapeutic treatment at the same 100 mg/kg daily level as the corresponding oral prophylactic group.

Consequently, these monkeys received 81.5 mg/kg for each of 4 successive days. Following treatment, all monkeys were carefully observed for overt signs of toxicity.

Beginning 25 days after exposure and continuing until termination of the experiment, feces from each animal were collected at weekly or biweekly intervals, were concentrated by the formalin-ether-buffered alcohol technique and fecal egg excretions were quantitated in terms of the numbers of eggs per gram of feces (NEPGF). Following intramuscular administration of 30 mg ketamine hydrochloride (Vetalar<sup>R</sup>) anesthetic and exsanguination by cardiac puncture, worms were recovered by perfusion with citrated saline. The liver and mesenteries of each animal were perfused separately and the worms were collected on a filtration apparatus as previously described. Tissue specimens were collected for histopathologic examination. The following criteria were used to evaluate drug effectiveness: the number and time of appearance of eggs in the feces; the number, sex ratio, appearance, and anatomic location of worms in the host; and the histopathologic manifestations of disease, especially in the liver and colon.

The untreated monkeys and those receiving therapeutic treatment on day 50 presented overt symptoms of illness during the sixth and seventh weeks after exposure. These consisted primarily of lethargy, anorexia and diarrhea with occult blood and mucus in the stools. This "crisis" period lasted no more than two weeks and had subsided by the eighth week post-exposure in all monkeys, including the untreated controls.

TABLE XV

Effect of Exposure Pattern upon Subsequent Transfer of I:mmunity by Cells

Exposure interval <sup>a</sup>	Number of worms <sup>b</sup>	Pe
	194 ± 35 189 ± 24	>0.05
$\begin{array}{ccc} 14 & - \\ - & 3 \\ 14 & 3^d \end{array}$	$169 \pm 24$ $103 \pm 13$ $169 \pm 19$	<0.05 >0.05

 Interval between initial exposure to cercariae and subsequent sacrifice of animal to obtain cells subsequently used for adoptive transfer of resistance.

 $^{b}$  Number of worms recovered from syngeneic animals which were injected iv with  $3 \times 10^{8}$  nonadherent peritoneal exudate cells, concomitant with exposure to cercariae and perfused 21 days later.

<sup>c</sup> P value vs number of worms obtained from animals injected with cells obtained from animals not previously exposed to S. mansoni.

d Animals exposed to S. mansoni 14 weeks and again 3 weeks prior to sacrifice.

Table XVI

IA-4 N-oxide treatment of rhesus monkeys infected with S. mansoni

				Treatment	ment	
Monkey No.	Weight (kg)	Type*	Route*	Days pre- or post-exposure	Orug dose per administration**	Total drug administered
P118 M830	3.5	۵	M	7	200	200
P257 P421	3.8	۵	8	-1, 0, 1, 2	100	400
P141 P704	5.1	-	MI	20	200	200
P429 P702	3.7	-	0	50, 51, 52, 53	81.5	326
P123 P135	3.4	<b>5</b>	1	1	1	1

Table XVII

Egg excretion and worm recovery data in rhesus monkeys infected with S. mansoni and treated with IA-4 N-oxide

		Egg excr	Egg excretion data	res l					Worm recovery data	data
Monkey		First eggs	NEPGF**	*	Se	Sex distribution	ribu	tion	Organ distribution	oution
No.	Treatment*	(day)	Mean***	Max.	Σ	ш	~	Total	Mesenteries	Liver
P118 M830	P-IM P-IM	76 55	<b>₽</b>	ر 20	16	0	04	35	30	0.5
P257 P421	P-P0 P-P0	48	21 16	72	16	15.8	00	24 40	30	40
P141 P704	MI-T	34	97	556 312	00	22	-0	82	3 ND***-	١
P429 P702	1-P0 1-P0	34	126 83	908	00	0-	00	0-	0-	00
P123 P135	22	34	288 523	624 932	189	182	30	371	369	2

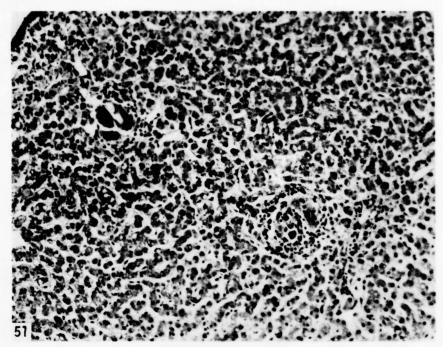


Figure 51. Liver, untreated control monkey. Two granulomas are present, one of which contains a schistosome egg with little surrounding host reaction.

Prophylactically treated animals presented no clinical disease manifestations.

Following drug administration, one therapeutically treated monkey (No. P141) developed a draining abscess at the site of intramuscular injection. This animal responded well to penicillin/streptomycin therapy and the lesion was resolved within one week. All other treated animals tolerated the drug extremely well and presented no symptoms of toxicity other than tissue irritation at the site of intramuscular injection.

The patterns of fecal egg excretion and worm burden at the time of necropsy (Table XVII) illustrate the efficacy of prophylactic and therapeutic treatments. Untreated control monkeys presented typically fluctuating but high egg counts (NEPGF) from 6 weeks post-exposure until termination of the experiment; the counts of therapeutically treated animals were reduced dramatically to zero by 12 days post-treatment. Fecal egg excretion from these animals remained essentially negative until the animals were necropsied. This effect was not seen in prophylactially treated monkeys, where the onset of patency was delayed but not prevented. In orally treated animals of this group, eggs appeared in the feces 2 weeks after that of control or therapeutically treated

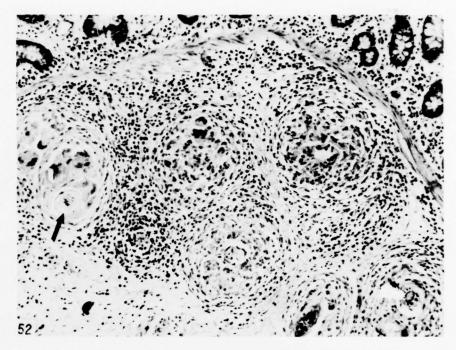


Figure 52. Colon. Several granulomas contain intact, probably viable eggs.

monkeys. In the animals receiving a single intramuscular administration of the drug the day before exposure, eggs were not seen until 3 weeks (No. M830) or 6 weeks (No. Pll8) after the control animals became positive. In neither case, however, did the total number exceed 72 EPGF before necropsy of the animals on day 76 post-exposure.

Worm burden analysis in the individual animals correlated well with fecal egg excretion data. Therapeutically treated monkeys harbored very few, if any, all of which were severely stunted. Conversely, oral prophylactic adminstration was the least effective treatment and these monkeys contained mature, paired schistosomes, although in much lower total numbers than those of the untreated control monkeys. Of the two animals receiving a prophylactic intramuscular administration, one (No. Pl18) had only 2 stunted male worms while the other (No. M830) had a worm burden consistent with those receiving oral prophylactic treatment. Most worms were recovered from the mesenteric circulation and could not be correlated with an hepatic shift as a result of treatment.

Necropsy. Monkeys from the control group had typical lesions associated with  $\underline{S}$ . mansoni infection. The colons were thickened with multiple, elevated hyperemic foci on the mucosal surface. Grossly there were small erosions present but intraluminal hemorrhage was not a feature at the time

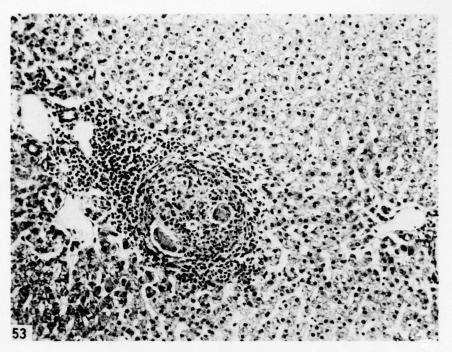


Figure 53. Liver, prophylyactically treated monkey. A single granuloma obliterates a portal triad and illustrates an intense host reaction with the presence of several giant cells.

of necropsy. Livers taken from control monkeys had many randomly distributed pale foci within the parenchyma. There was nos gross evidence of portal fibrosis in these animals. The mesenteric lymph nodes draining the large intestine were moderately enlarged.

Prophylactically treated monkeys had lesions similar to the controls but the number of such lesions and their gross appearance suggested a much less severe disease. The monkeys from the therapeutically treated groups had essentially normal appearing livers and colons.

<u>Histopathology</u>. Sections of liver and colon from control monkeys had many active granulomas characterized by the presence of schistosome ova, large multinucleate gaint cells and an admixture of eosinophils and mononuclear inflammatory cells (Figures 51 and 52). Ova present ranged from apparently intact viable ova to degenerating fragments and debris. There was a mild degree of fibroplasia associated with most of the granulomas.

Orophylactically treated monkeys had fewer granulomas present in the liver and colon and many of these lesions appeared to be in the resolving stage (Figures 53 and 54). Apparently viable ova were present in a few granulomas indicating active infection. The therapeutically treated

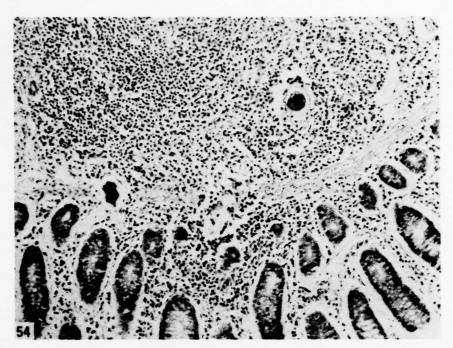


Figure 54. Colon, prophylactically treated monkey. Apparently viable ova are present in the submucosa and muscularis mucosa, but host reaction is minimal.

groups had essentially normal livers with only scattered foci of mononuclear cells and a few nearly healed granulomas (Figures 54 and 55). One monkey (No. P429) had several resolving granulomas in the colon, none of which contained viable ova. All of the other monkeys in this group had histologically essentially normal colons.

IA-4 N-oxide was well tolerated orally with no signs of nausea or discomfort and no emesis. All animals receiving the drug via intramusclar injection experienced tissue irritation and one developed an abscess.

Control monkeys and therapeutically treated monkeys prior to therapy exhibited typical manifestations of overt schistosomiasis infection, i.e., lethargy, anorexia, and diarrhea with blood, mucus and high egg counts. Necropsy and histopathologic findings of control monkeys were consistent with subacute, unresolved schistosomiasis.

Prophylactic results indicate a 2 to 6 week delay in patency and egg excretion levels remaining well below those observed in control monkeys. The low egg excretion levels were confirmed on necropsy by the significantly lower worm recovery when compared with controls, the marked

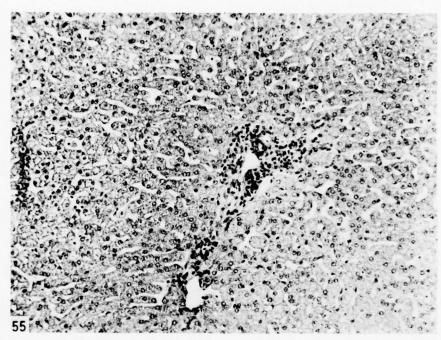


Figure 55. Liver, therapeutically treated monkey. A nonspecific portal infiltrate of lymphocytes, macrophages and a few neutrophils are the only remaining evidence of the cleared infection.

decrease in severity of lesions, and the limited number of hepatic and intestinal granulomas. Intramuscular administration appeared to be more effective than oral treatment, as shown by the longer delay in patency and lower numbers in egg excretion data. The difference observed in the number and condition of worms recovered on necropsy is probably not significant in light of the limited number of animals involved. The failure to completely inhibit worm development dictates against use of this drug in a prophylactic mode because of the potential for development of drug resistance. Such resistance has been demonstrated in mice for hycanthone and has also been implicated as the reason for failures of hycanthone therapy of human schistosomiasis.

Therapeutic efficacy of IA-4 N-oxide was outstanding by either oral or intramuscular routes of administration. There appears to be no significant difference in effectiveness between the two routes under the test conditions. Egg counts showed a dramatic drop by 12 days post-treatment and all animals quickly reached a zero level. No normal worms were recovered on necropsy and the resolving granulomas and absence of viable ggs on histopathologic examination confirmed the cures. Monkey P429, which had the highest egg excretion level of all 10 animals by the 7th week, had no worms at necropsy.

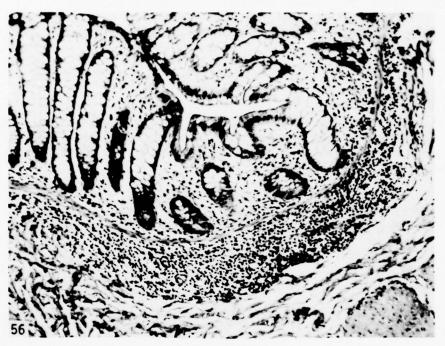


Figure 56. Colon, therapeutically treated monkey. A diffuse accumulation of lymphocytes remains in the submucosa and a few lymphocytes are still present in the lamina propria.

Considering 1 the incomplete prophylactic effect of IA-4 N-oxide, 2) the potential of this phenomenon for influencing the subsequent development of drug resistance, and 3) the tissue irritation associated with the intramuscular route of administration, further evaluation might best be concentrated on the oral therapuetic approach. At the same time, efforts should be initiated to more clearly define the potential for teratogenic or carcinogenic effects.

Project 3M161102BS01 BASIC RESEARCH ON MILITARY DISEASES

Work Unit 129 Parasitic Diseases of Military Importance

### Literature Cited.

#### Publications:

- 1. Phillips, S. M. DiConza, J. J., Gold, M. A. and Reid, W. A.: Schistosomiasis in the congenitally athymic (nude) mouse I. Thymic depedency of eosinophilia, granuloma formation, and host morbidity. J. Immun. 118: 594-599, 1977.
- 2. Phillips, S. M., Reid, W. A. and Sadun, E. H.: The cellular and humoral immune response to <u>Schistosoma mansoni</u> infections in inbred rats II. Mechanisms during reexposure. Cellular Imm. 28: 75-79, 1977.
- 3. Redington, B. C. and Hockmeyer, W. T.: A method for estimating blood meal volume in Aedes aegypti using a radioisotope. J. Insect Physiol. 22: 961-966, 1976.
- 4. Reid, W. A. and von Lichtenberg, F.: Experimental Schistosoma japonicum infection in miniature pigs. J. Parasit. 63: 392-394, 1977.
- 5. Reid, W. A., Phillips, S. M. and Roscinski, R. J.: Schistosoma mansoni: Radioisotope uptake and retention by cercariae and developing schistosomules. Exp. Parasit. 42: 331-342, 1977.
- 6. Reid, W. A. and Reardon, M. J.: Mesocestoides in the baboon and its development in laboratory animals. J. Med. Primatol. 5: 345-352, 1976.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY					OA 6441	77 10			CONTROL SYMBOL R&E(AR)636
76 10 01	D. Change	5. SUMMARY SCTY	WORK SECURITY		NA	NL	ON SPECIFIC	R ACCESS	S. LEVEL OF SUM  A. WORK UNIT
10. NO./CODES:®	PROGRAM ELEMENT	PROJECT	NUMBER	TASK	AREA NUMBER			IT NUMBER	
& PRIMARY	61102A	3M161102BS	:01		00		130		
b. CONTRIBUTING	OTTOER	O TO	<u> </u>						
с. СДОХИДОХИСК	CARDS 114F								
	Security Classification Code								
(U) Viral	Infections of	Man							
	CHNOLOGICAL AREAS								
002600 Biol	ogy 010100 M	icrobiology	003500 C1	linic	al Medici	ne	Tie Benen	MANCE MET	
					1	1			
63 08		I CONT		DA	OURCES ESTIMAT		IONAL MAN Y	In-Hou	SE (In thousands)
A DATES/EFFECTIVE:	NA	EXPIRATION:		10 MES	PRECEDING	E PROFESS	TONAL MAN Y	RS & FUR	US (In thousands)
L NUMBER:	MA.			FISCAL	77		1.5		448
C TYPE:		& AMOUNT:		YEAR	CURRENT		1.5		340
& KIND OF AWARD:		f. CUM. AMT.			78		1.5		177
19. RESPONSIBLE DOD	ORGANIZATION			20. PER	FORMING ORGANI				
wame: Walter	Reed Army In	stitute of	Research	HAME:*	Walter R	eed Army	Insti	tute o	f Research
Div of CD&I									
ADDRESS:* Washington, D.C. 20012 ADDRESS:* Washington, D.C. 20012									
PRINCIPAL INVESTIGATOR (Puminh 88AN II U.S. Academic Institution)								,	
RESPONSIBLE INDIVIDUAL NAME: BANCROFT, LTC William H.									
NAME: RAPMUND, COL Garrison TELEPHONE: (202) 576-3757									
TELEPHONE: (202) 576-3551 SOCIAL SECURITY ACCOUNT NUMBER:									
ASSOCIATE INVESTIGATORS  HAME: TOP, COL Franklin H., Jr.									
Foreign in	ntelligence n	ot consider	ed		DDANDT	D. 11-1	A F		
22. KEYWORDS (Frecedo	BACH with Society Classifi	cetton Code) (11)	Vinusos: 1	111 7	DRANUT.	- /11) A	ter E.	. I.f.	
(11) Adenovi	rus Respirato	ry Disassas	· (11) Infl	U) II	iniuno rogy	man Volu	ntoon	s Inte	ctions;
23. TECHNICAL OBJECT	efine etiolog	PROGRESS (Fumial in	dividual paragrapho Id	dentified by	number. Precede I	ext of each with	ocurity Closel	Reatlan Code	4
23 (U) 10 de	to determine	y of acute	infectious	infl	eases of	special	nazard	to mi	intary
severity and	d medical res	ult of huma	n virus in	fect	ions, and	to deve	lop me	ans fo	r re-
	bility due to								
24 (U) Conte	emporary viro	logical and	immunolog	ical	methods	are appl	lied to	disea	se
problems occ	curring in tro reas. New co	oops or in	susceptibl	e ci	vilian po	pulation	ns in s	trateg	ically
important a	reas. New co	nceptual ap	proaches a	ind me	ethods ar	e develo	pped as	neede	d for
specific pro		out mucoc	Infaction	of h	ıman nhaq	ocutic n	onocut	oc wit	h donauo
virus in-vit	0 - 77 09 Arbo tro was found	to correla	to with vi	rule	inan phay	ild stra	in of	denque	type 2
	produced both								
	A small plage								
	little, if at								
	ccessfully to								
	low levels o								
	valuating the								
for virus id	dentification	was found	to have ma	ximur	n titers	of neutr	ralizin	g anti	body when
purified who	ole virus was	separated	from nonin	fect	ious vira	1 protei	ins. R	outine	partial
	of viruses								
was isolated	d during an e	pidemic in	Jamaica. T	he in	napparent	:apparer	it infe	ction	ratio for
	lly susceptib								
	re recovered								liminary
	ions include								0 6 77
ee walter i	Reed Army Ins	titute of R		inual	Progress	Report	Jul	/6 - 3	U Sep //.
*Aveilable to contract	tore upon originator's appr	ovel.	105						
DD FORM 149		EDITIONS OF TH	S FORM ARE O	BSOLE	E. DD FORM	5 1498A, 1 N	OV 68		
MAR 68	AND 1498-1	. I MAR 66 (FOR	ARMY USE! ARE	09301					

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY					DA OA 6441 77 10 01 DD-DR&E					
S. DATE PREV SUM'RY	A. KIND OF SUMMARY	S. SUMMARY SCTY	S. WORK SECURITY	7. REGRA		DA DH	NL	ON TRACTO		A SORK UNIT
76 10 01	D Change		NUMBER		REA NUME	_	INL		T NUMBER	
PRIMARY	61102A	3M161102B		TASK A	00	ER		130	TNUMBER	
b. CONTRIBUTING	OTTOEN	3111011020	301	1-	00	-		130		
**************************************	CARDS 114F			1		-				
	Security Classification Code					_				
(U) Viral I	nfections of	Man								
002600 Biol	ogy 010100 Mi	crobiology	DO3500 CT	inica	Medi	<u>cin</u>	<u>e</u>	14 PERFOR	MANCE WET	100
63 08		CONT		DA	DURCES EST		<b></b>		In-Hou	
& DATES/EFFECTIVE	NA	EXPIRATION:		10. RESC	PRECEDIN		& PROFES	SIONAL MAN Y	ES E FUN	OS (In thousands)
b. NUMBER:*				FISCAL			1			
G TYPE:		& AMOUNT:		YEAR	CURRENT		1		_	
& KIND OF AWARD:		f. CUM. AMT							1	
19 RESPONSIBLE DOD	ORGANIZATION				ORMING OR					
MAME: Walter	Reed Army In	stitute of	Research	HAME:*	Walte Div o	r R f C	eed Arm D&I	y Insti	tute o	f Research
ADDRESS: Wash	ington, D.C.	20012		,				. 2001		
PRINCIPAL INVESTIGATOR (Pumieh MAN II U.S. Academic Institution)										
RESPONSIBLE INDIVIDA	JAL			NAME:				, Willi		
NAME: RAPMUND, COL Garrison					-	202	) 576-3	757		
TELEPHONE: (202) 576-3551 SOCIAL SECURITY ACCOUNT NUMBER										
21. GENERAL USE				ASSOCIA	TE INVEST	GATOR	15			
Foreign in	telligence no	t consider	ed	HAME:	DOAM	DT	On Ma	lin H., Iter E.		
(U) Adenovi	rus Respirato	rv Disease	Viruses;	(U) Ir	muno1	vpo	; (U) A	rboviru	s Infe	ctions;
23. TECHNICAL OBJECT	TIVE. 24 APPROACH, 25	PROGRESS (Pumleh	Individual perseraphe Id	fentitled by	number. Pro		coocial	Socurity Classif	to mi	Sitanu
23 (0) 10 0	efine etiolog to determine	y or acute	to factors	infl	eases	01	Special	ca dic	tribut	ion
coverity an	d medical res	ult of hum	an virus in	ofect	ions	and	to dev	elon me	ans fo	r re-
	bility due to			HECL	, ,	anu	to dev	e rop me	uns 10	
	emporary viro			nical	metho	ds	are app	lied to	disea	se
problems oc	curring in tr	oops or in	susceptib	le ci	vilian	DO	pulatio	ns in s	trateq	ically
important a	reas. New co	nceptual a	pproaches a	and me	ethods	ar	e devel	oped as	neede	d for
specific pr										
25 (U) 76 1	10 - 77 09 Ade	enovirus.	Adenovirus	type	21 W	as 1	the pre	dominant	cause	e of acute
respiratory	disease (ARD)	in basic	combat tra	inees	. Dis	seas	se rates	stayed	below	w 3.0/100/
	posts. A fie									
	ation with the									
	ted a determin									
izing antic	ody detection	was comp	licated by	a sud	den no	ona	vailabi	lity of	prima	ry human
emoryonic ki	idney cells.	Alternati	ve tissue c	ultur	e cei	1 1	ines and	a hema	igglut	ination
test were th	ried for antil	ody detec	tion withou	t suc	cess;	ет	forts a	re now t	eing	irected
for monitori	loping a radio ing influenza	infaction	ay. Influe	comba	t tra	inc.	oc at E	system	Was II	on and
Knox. Infl	uenza A simil	ar to A/Vi	ctoria/75	DUIID O	COVO	od	from al	1 posts	and di	nfluor
B/HK from F	t. Jackson.	Cytomegalo	virus info	tion	of so	na1	thanch	lant na	tionts	was four
to contribu	te to increas	ed nationt	mortality	hu+ *	of re	nal	araft	failune	For	technical
report. see	Walter Reed	Army Incti	tute of Por	parch	Annu	al	Progres	s Penon	1 1 1	1 76 -
BO Sep 77.	na ruer need	THIS THIS CT	tute of kes	earci	- Amiru	aı	riogres	s vehou.	c i ou	. 70 -
			108							
	lors upon originator's appr	ovel.								
DD FORM 149	PREVIOUS	EDITIONS OF T	HIS FORM ARE O	BSOLET E OBSOL	ETE.	ORM	1498A, 1 P	10 V 68		

Project 3M161102BS01 BASIC RESEARCH ON MILITARY DISEASES

Work Unit 130, Viral Infections of Man

Investigators:

Principal: LTC W.H. Bancroft, MC; W.E. Brandt, Ph.D.

Associate: COL P.K. Russell, MC; COL F.H. Top, Jr, MC; MAJ D.S. Burke, MC; MAJ K.E. Dixon, MC;

MAJ D.S. Burke, MC; MAJ R.E. DIXON, MC; MAJ K.J. Winn, MC; MAJ B.W. Booth, MC; CPT R.G. Allen, MSC; J.M. Dalrymple, Ph.D.;

A.B. White, Ph.D.; SSG D.A. Leach; SP6 B.H. Robinson; SP5 P.J. Engineri; SP5 K.J. Belleville; SP5 L.D. Stovall; SP5 E.M. McDowell; SP4 S.M. Crump; SP4 J.R. Putnak; SP4 L.C. Webster;

SP4 D.A. Fraser; J.M. McCown; B.H. Mann; S.A. Ralph; H.G. Cannon; R.J. Jackson

G.P. Onley

## Description

To define the etiology and ecology of human virus infections, particularly those of military medical significance; to devise and evaluate means for precise diagnosis, control and/or prevention of disease. Studies have applied virological, immunological, epidemiological and physiological approaches to understanding disease caused by respiratory, arthropod-borne and other viruses, the factors influencing transmission among men, other vertebrates and invertebrates, and their survival in nature.

#### Progress

- I. The arthropod-borne viruses
- A. Replication of dengue viruses in human monocytes as a new laboratory marker for virulence

In previous collaborative studies (Theofilopoulos et al, 1976) it was shown that dengue-2 virus adapted to Raji cells, a continous human lymphoblastoid cell line (Sung et al, 1975) and replicated readily in monocytes from normal (non-dengue immune) human donors. It had been thought that potent dengue replication would occur only in monocytes if they were obtained from an individual who had experienced

a dengue infection, generally with one of the heterologous serotypes or if non-neutralizing antibody was added to the culture system. In this report we show that 1) wild dengue maintained in certified primary cells rather than in transformed or malignant cells can replicate to a relatively high titer in normal human monocytes without added antibody; 2) that these monocytes can be infected within 24 hours of collection, whereas lymphocytes are not susceptible to dengue unless the virus if first adapted to Raji cells and the lymphocytes are cultured for 3 days before they are susceptible to infection; 3) when virus replicates to titers of less than 103 plaque forming units (PFU)/ml, heterologous or dilute homologous antibody included in the culture medium will enhance virus titers, up to 200-fold; 4) antibody depresses, rather than enhances, Raji adapted dengue virus replication in lymphocytes; 5) virus replication can result in titers of 106 PFU/ml in monocytes from normal donors, which cannot be improved with added antibody; 6) normal monocytes that replicate wild virus will not replicate a candidate dengue-2 live virus vaccine strain; 7) monocytes from a dengue immune donor or the presence of antibody in the culture medium with normal monocytes, will allow a delayed and low level replication of the dengue-2 vaccine strain, similar to the pattern of replication observed with the attenuated yellow fever vaccine.

## Isolation of monocytes and lymphocytes

Since the relatively high levels of dengue virus replication in monocytes from normal human donors does not agree with published information from another laboratory (Halstead et al, 1973, 1976), the following description of the monocytes isolation procedure is given:

Blood was generally drawn in four 60ml aliquots of 50 ml syringes containing 1 ml of 1000 unit/ml heparin. Each 60 ml of heparinized blood was immediately delivered into a 75 cm² plastic culture flask (Falcon 3024) containing 75 ml of blood diluent (Ca++ and Mg++ free Hanks balanced salt solution with 0.01 M Hepes buffer and 0.0025 M EDTA). After gentle mixing, the diluted blood was dispensed into 50 ml plastic conical centrifuge tubes (Falcon 2070) in 35 ml volumes. Approximately 15 ml of Ficoll-Hypaque, specific gravity 1.077, was carefully injected beneath the diluted blood with a spinal needle. The Ficoll-Hypaque was prepared by mixing 450 ml of 9.0% Ficoll (Pharmacia or Sigma) with 187.5 ml of 33.9% hypaque previously diluted down from a commerical 50% solution (Sterling Organic). The specific gravity was adjusted from 1.082 to 1.077 with about 50 ml of distilled water. For convenience, Ficoll-Hypaque solutions of 1.077 and 1.082 prepared according to Boyum (1968) were measured in a refractometer; subsequent batches were then adjusted according to the refractive index.

The tubes containing the diluted blood on top of a Ficoll-Hypaque cushion were centrifuged at  $350 \times g$  for 40 minutes at room temperature.

White cell layers visible just above the Ficoll-Hypaque were aspirated in 12 to 13 ml volumes with a 10 ml pipette, pooled, diluted 1:2 with blood diluent, and centrifuged again at 350 x g for only 7 minutes at 4C. The white cell pellets were resuspended in 100 ml of blood diluent and recentrifuged for 7 minutes two more times.

The final white cell pellets were suspended in 10 ml of growth medium for enumeration of cells. Growth medium consisted of RPMI 1640 containing 10% fetal calf serum and 0.01 M Hepes buffer. Microscopic examination revealed mononuclear phagocytes (monocytes) and lymphocytes. The following table shows that the yield of white cells ranged from 2.0 to 6.6 x 108/240 ml blood.

TABLE 1. Yield of white cells obtained from 240 ml blood from human donors in order of experiments.

Donor	Yield x 10 <sup>8</sup>	Donor	Yield x 10 <sup>8</sup>	Donor	Yield x 108
A.B.	2.6	J.D	3.4	B.J.	3.5
C.B.	2.2	K.B.	2.4	B.P.	2.0
L.A.	3.2	M.W.	2.4	B.P.	2.4
L.A.	2.8	W.B.	2.0	D.F.	4.4
D.P.	4.8	J.D.	3.5	L.W.	3.8
Z.T.	4.0	M.W.	4.5	R.S.	2.7
B.P.	4.0	F.M.	6.0	J.D.	6.6
S.H.	3.4	S.H.	2.5		

Utilizing the separation method described in our collaborative studies (Theofilopoulos et al, 1976) the white cells were plated in 20 ml growth medium at  $1.3 \times 10^7$  in cell culture petri dishes (Falcon) having a surface area of 50 cm<sup>2</sup>. The dishes were incubated at 35 C in a CO2 humidified incubator for 3 hours, then rotated to resuspend the nonadherent cells, which were aspirated from the plates and stored in plastic  $75 \text{ cm}^2$  cell culture flasks. The plates were washed with 5 mlblood diluent to remove residual nonadherent cells as well as loosely adherent cells. The adherent cells were scraped from the plastic surface with a rubber policeman in 0.5 ml blood diluent pooled and counted; approximately 10 to 15 percent of the total white cell population was obtained at this step, and they are referred to as "originally adherent cells". These cells were plated again at 5 x 106 per 25 cm2 plastic cell culture flask (Falcon 3012) and incubated with the caps loose in the CO2 incubator overnight. The following morning the growth surfaces of the flasks were washed to remove nonadherent cells. The final adherent cell population (secondary adherent cells) represented about I percent of the

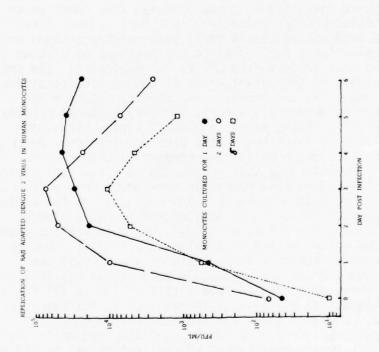
starting white cell population and served as the major cell type for the study of dengue infection. The adherent cells had the characteristic morphology of mononuclear phagocytes and retained their ability to phagocytize latex particles.

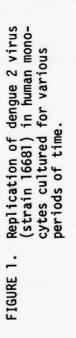
2. Effect of virus passage history and monocyte culture time on susceptibility to infection.

The susceptibility of the secondary adherent cells to dengue virus was first evaluated with the 16681 strain of dengue-2 virus, isolated from a case of dengue hemorrhagic fever in Thailand, and adapted to grow in Raji cells. Aliquots of cells in their first, second and fifth days of culture were infected at a theoretical multiplicity of infection (MOI) of 0.2 ( $10^5$  infectious units in 1 ml spread over 5 x  $10^5$  monocytes scattered over the plastic surface). Virus in the inoculum was allowed to adsorb to the cells for  $1^1_2$  to 2 hours at 35 C after which the inoculum was removed and the plastic growth surface gently washed to remove residual inoculum. Six milliliters of growth medium was added to the flasks, 1 ml was removed for a 0 time sample and the flasks were incubated for 5 to 6 days. One milliliter of medium was removed at daily intervals and replaced with an equal amount of fresh medium. The timed samples were assayed for infectious virus by plaque formation on LLC-MK2 cells (Eckels et al, 1977).

Monocytes infected during their first day in culture produced peak virus (in excess of 104 PFU/ml) for a longer period of time than the cells infected during their second day in culture (Figure 1). Second day cells appeared to produce virus sooner and to slightly higher titers than the day l culture cells, but virus production fell below the day l cells on the last 3 days of the 6 day experiment. Monocytes infected during their fifth day in culture also replicated dengue virus, but only to a low titer. Nevertheless, the quantities of virus that were obtained indicated that monocytes could be infected productively anytime after isolation.

The quantities of virus produced by a culture of monocytes at any given time post infection was not consistent even in cells from the same donor obtained only one month later (Figure 2). It can be seen in this figure that virus production in monocytes (infected during the first day of culture) was still climbing by the fifth day post infection, whereas in Figure 1 at this time, virus production had reached a plateau or was decreasing. The ability of dengue virus to replicate in human monocytes may depend on the strain or passage history as described in our previous study (Theofilopoulos et al, 1976). Figure 2 shows the growth curve of the Raji adapted virus strain 16681 after it was passaged once in LLC-MK2 cells; the virus replicated about as well as before it was passaged in LLC-MK2 cells. However, a potent mouse brain seed of dengue 2 virus did not replicate, even when it was passed once in LLC-MK2 cells





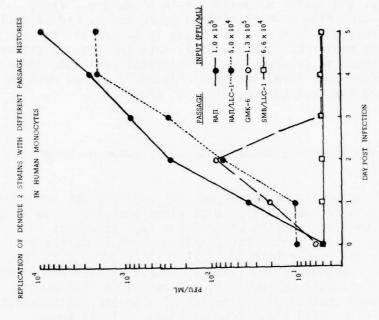


FIGURE 2. Replication of dengue 2 virus strains in monocytes from the same donor as was in Figure 1 about 1 month later.

(Figure 2). Finally, the Puerto Rico-159 strain of dengue 2 virus passaged 6 times in certified primary african green monkey kidney cells (GMK-6) was only detected 2 days post infection in monocytes from the same donor. Monocyte donors consistently fell into two groups; either a small amount of virus was produced by the monocytes from some donors (always detected 2 days post infection as in Figure 2) or, as much as 106 PFU/ml similar to the Raji adapted virus in Figure 1.

3. Growth of wild and attenuated dengue viruses on human monocytes.

Background and purpose: One of our current goals is the isolation and passage of variants or subpopulations of virus particles from wild (parent) dengue viruses representing each of the four serotypes to obtain variants might be attenuated for possible human immunization. The ultimate goal is the preparation of a multivalent vaccine to the dengue viruses. The precedent for a live flavivirus vaccine was established with the serologically related yellow fever virus. Laboratory work is directed toward establishing a series of characteristics which clearly differentiate the wild virus from a cloned variant; these characteristics may provide an indication as to the suitability of the cloned variant(s) for use as a candidate live virus vaccine.

The parent dengue-2 virus (PR-159) was isolated from human serum and passaged in certified primary grivet monkey kidney cells 6 times (GMK-6); the viruses produces large and small plaques when assayed on LLC-MK2 cells. A small plaque forming clone was isolated by picking plaques and passaging them on certified GMK cells monolayers. The small plaque clone, in contrast to the parent virus is temperature sensitive, a virulent for suckling mice, and produces an antibody response in monkeys without producing detectable viremia (1972-1976 Annual Reports). We studied the interaction of the parent and small plaque virus with human monocytes isolated as described in the previous section. We also studied the phenomenon of immunological enhancement described by Halstead et al., (1976, 1977) who indicated that potent dengue virus replication would occur only in leukocytes from normal or non-immune donors if heterologous or homologous non-neutralizing antibody was added to the culture medium (i.e. using a 1:5000 dilution of antiserum that had a plaque reduction neutralization titer (PRNT) of 1:80). Our observations with primate monocytes (see below) agreed with Halstead's findings, but we found that human monocytes could replicate wild dengue without the addition of antibody to the culture system. However, when replication in some monocyte cultures only reached peak titers of 103 to 104 PFU/ml, antibody added to aliquots of the same cells resulted in increases of virus up to 400-fold. Whenever the virus replicated as high as 106 PFU/ml in monocyte cultures, from some donors, antibody had no further enhancing effect. This immune enhancement of replication was tested on both the parent and small plaque vaccine candidate.

a. Laboratory production of parent and small plaque viruses. Both viruses grow very well in certain laboratory cell culture systems. Figure 3 illustrates the growth curves of both viruses in aliquots of LLC-MK2 cells infected with and cultured in normal medium (10% FBS in medium 199) containing either normal human serum at a 1:20 dilution, a 1:20 dilution of heterologous dengue-3 human antiserum (DEN-3 PRNT = 1:20), or a 1:500 dilution of homologous dengue-2 human antiserum (Den-2 PRNT = 1:80). Each of the viruses replicated equally well in cells cultured in each medium, reaching peak titers of about 106 PFU/ml. Thus, the presence of antibody had no effect on the relatively potent LLC-MK2 replication system and immune enhancement generally cannot be demonstrated.

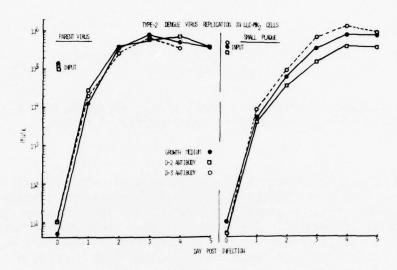


FIGURE 3. Replication of parent (left graph) and small plaque (right graph) dengue-2 (PR-159) viruses in LLC-MK2 cells cultured in either normal or antibody containing medium.

b. Replication of parent and small plaque viruses in monocytes. A distinctly different comparative pattern was found when these two viruses were tested for their ability to replicate human monocytes. Figure 4 depicts the growth curves of the parent and small plaque dengue-2 (PR-159) viruses in monocytes from two different donors. This is representative of the situation we observed with the small plaque vaccine candidate in normal monocytes; either a small amount of virus was detected 2 days post infection after which no virus is found, or, no virus was detected at all. The parent virus, on the other hand, generally replicates to at least 10<sup>3</sup> PFU/ml. We found one donor out of 16 whose monocytes would not replicate the parent virus at all, and another donor whose monocytes only replicated the parent virus to 10<sup>2</sup> PFU/ml (shown below).

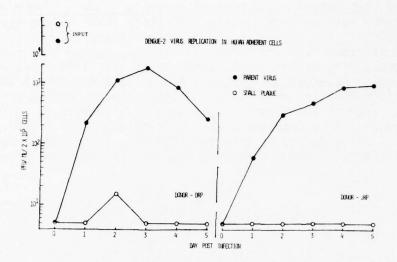


FIGURE 4. Replication of parent and small plaque dengue-2 (PR-159) viruses in human monocytes from 2 different donors.

As stated above, the parent virus produces a mixture of small and large plaques on monolayers of LLC-MK2 cells; however, after passage or replication in monocytes, we usually observe mainly large plaques in the LLC-MK2 assyas (Figure 5). The monocytes thus seem to restrict the replication of the small plaque forming dengue viruses, whether they be present in the parent virus at the 6th GMK passage level, or as a purified small plaque seed at the 19th passage level.

c. Replication of the parent and small plaque viruses in normal monocytes cultured in the presence of non-neutralizing antibody. Both viruses were mixed with media containing either normal sera, heterologous dengue-3 antibody, or homologous dengue-2 antibody dilutions as described in section a. The mixtures were used to infect the cells, and, after removing the inoculum and washing the adherent cells free of residual inoculum, medium containing the same antiserum dilution was placed on the monocytes. One milliter of medium was removed immediately and at daily intervals and the same volume of fresh medium was added back to the flasks. The results of plaque assays on these samples is depicted in Figure 6; the parent virus did not replicate very well in monocytes cultured in normal medium (less than 102 PFU/ml at 2 days post infection). However, the same virus replicated to titers up to 200-fold greater by the third day post infection when dengue antibody was included in the culture medium. This confirms the observation of immune enhancement described by Halstead and O'Rourke (1977) who worked with unfractionated peripheral blood leukocytes.

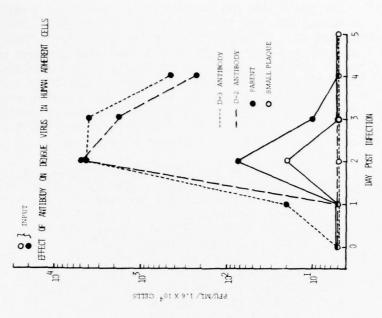


FIGURE 6. Immune enhancement of parent, but not small plaque, dengue-2 (PR-159) virus in human monocytes.

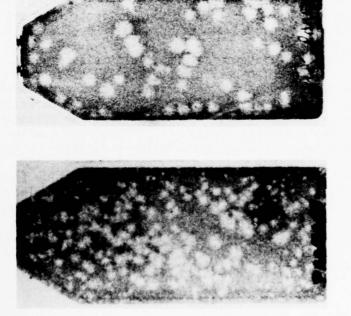


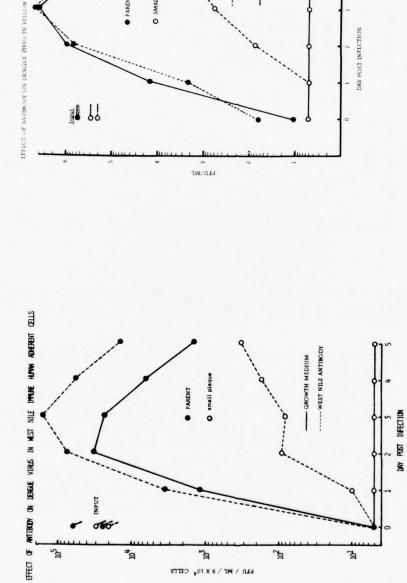
FIGURE 5. Dengue-2 parent virus passaged in monkey kidney cells (left) and in human monocytes (right).

Immunological enhancement was not observed with the small plaque vaccine candidate in monocytes from the same donor (Figure 6). Small plaque virus was detected at a slightly lower titer than the parent virus in normal medium 2 days post infection as it occurred with another donor in the previous figure. No virus was detected at all from monocytes infected with the small plaque virus in the presence of antibody. The titer (quantity) of the input virus used to infect the monocytes was the same after mixing it with either normal medium or antibody containing medium, about 2.5 x  $10^4$  PFU (Figure 6-top), similar to the input parent virus which replicated very well in the presence of antibody.

d. Replication of parent and small plaque viruses in monocytes from flavivirus immune donors. We studied the replication of the two viruses in monocytes from a foreign visitor who had recovered from a severe case of West Nile Fever in March of 1971. West Nile virus is serologically and structurally related to dengue virus, both being members of the flavivirus group of Togaviridae, (formerly the group B arboviruses). Aliquots of "West Nile sensitized" monocytes were infected with the dengue type 2 (PR-159) parent and small plaque viruses in the presence of normal medium and West Nile antibody at a final donor serum dilution of 1:20. (The HI titer of this serum was 1:320, the PRNT titer was >1:160 in 1971; the HI titer in 1974 was 1:160)

The dengue-2 parent virus replicated very well in the West Nile sensitized monocytes cultured in normal medium, and it replicated about 8-fold higher in these monocytes cultured in medium with West Nile antibody (Figure 7). The small plaque vaccine candidate, used at almost the same MOI as the parent virus, did not replicate at all in the West-Nile monocytes from the West Nile immune donor cultured in normal medium. However, in these monocytes cultured in West Nile antibody, the small plaque virus replicated in a delayed manner, gradually producing increasing quantities of virus through the 5 day culture period. A plaque assay revealed the presence of intermediate and large size plaques, rather than all small plaques, indicating that some reversion in plaque characteristics had occurred under the conditions of this experiment. Reversion can be prevented by adjusting the multiplicity of infection as indicated previously (Eckels et al, 1976). However, the presence of non-neutralizing antibody probably creates antigen-antibody complexes and more efficient infection via an Fc receptor on the monocyte.

Similar experiments were done with monocytes from a yellow fever immune donor (Figure 8) and a dengue immune donor (Figure 9). The small plaque virus either did not replicate at all or did not replicate well unless the monocytes were infected and cultured in the presence of non-neutralizing antibody. In the monocytes from both individuals, the parent virus replicated so well that little if any, immune enhancement was demonstrable with added antibody in the culture medium.



rg

PFU / ML / 9 X 104 CELLS

-GROWTH MEDIUM

----ANTIBODY - D-2

O SMALL PLAQUE

FEVER IMMUNE CELLS

105

104

Immune enhancement of both parent and small plaque dengue-2 (PR-159) viruses in human monocytes from a flavivirus (West Nile) immune donor. FIGURE 7.

Replication of dengue parent and small plaque viruses in monocytes from a yellow fever immune donor. FIGURE 8.

70

þ

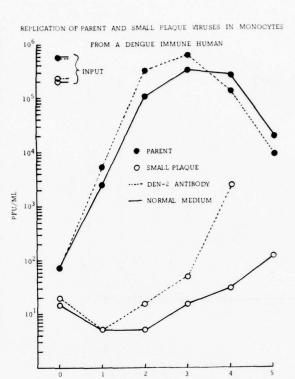


FIGURE 9. Replication of parent and small plaque dengue-2 (PR-159) viruses in human monocytes from a dengue-2 immune donor.

DAY POST INFECTION

We found one normal donor whose monocytes replicated the small plaque virus up to about 10<sup>3</sup> PFU/ml, but again, in a delayed manner (Figure 10). The presence of antibody increased the yield even more. Since the Asibi parent of the yellow fever vaccines can produce lethal infections in humans, the replication of two yellow fever vaccine viruses was compared with wild dengue virus as depicted in Figure 11. Again, the parent dengue virus replicated so well that immune enhancement could not be demonstrated. The yellow fever vaccine strains, on the other hand, replicated in a delayed manner with lower titers. However, the pattern of replication of the dengue-2 small plaque virus in Figure 10 appeared to be similar to that of the attenuated yellow fever live virus vaccine in monocytes from normal donors (Figure 11).

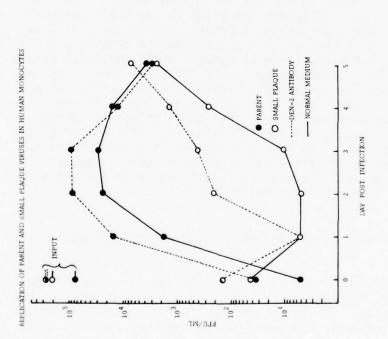


FIGURE 10. Immune enhancement of parent and small plaque dengue-2 (PR-159) viruses in monocytes from a normal donor. Small plaque virus replication was usually associated with the appearance of layer plaques.

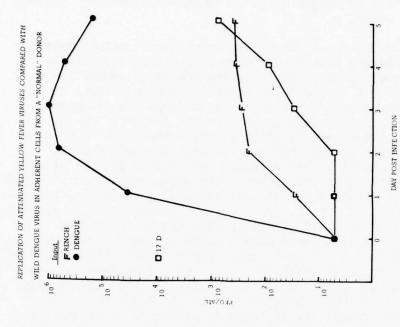


FIGURE 11. Replication of yellow fever vaccine strains (French mouse neurotropic and American egg passage 17D) compared to wild dengue virus.

4. Replication of dengue virus in human monocytes with varying adherence characteristics.

In order to determine if there was a functional requirement for monocytes to have adherent properties to replicate dengue virus, we attempted to obtain monocytes with increasing adherence properties by harvesting the cells that came off in increasing concentrations of ethanol. As described above, white cells isolated by Ficoll-Hypaque centrifugation were incubated in petri dishes for 3 hours. The non-adherent cells were removed and the plates were washed with 10 ml of blood diluent. The plates were then swirled with another 10 ml of blood diluent and those cells that detached were pooled and set aside as a "O" percent ethanol cell population. The dishes were then incubated for 5 minutes each time with blood diluent containing increasing concentrations of ethanol from 0.1 to 2.5 percent. Following the 2.5% alcohol wash, about 4 to 15% of the cells remained attached to the plates. The cells in the alcohol solutions were washed twice by centrifugation, counted and replated in 1.8 cm<sup>2</sup> wells in a Linbro 24-well tissue culture tray. Only 0.1% of the cells reattached after this procedure. These cells, in addition to the cells remaining adherent to the large dishes after the ethanol washes, were infected with the parent virus. The results of a 5 day experiment are depicted in Figure 12 which shows the growth curves adjusted PFU/m1/2600 cells, and sorted on two adjoining graphs. The family of curves on the left indicate that the less easily detachable cells, those detached only by increasing concentrations of ethanol (up to 0.8%), have a decreased capacity to replicate dengue-2 virus. The curves on the right graph in Figure 12 indicate that 2.5% alcohol removed some cells that could replicate dengue dengue, and finally those cells that were not susceptible to removal could replicate dengue even better. While the interpretation of the "ethanol" curves may not be valid because of the low percentage of cells that reattached after removal by the alcohol, the important point is that there is both a strongly and weakly adherent cell population that replicates dengue virus. The concept of weakly and strongly adherent cells may have some significance when replication in monkey monocytes is considered in the following section. We found that dengue replicates only in weakly adherent cells from monkeys, whereas both weakly adherent and strongly adherent human cells support dengue replication.

5. Replication of parent and small plaque dengue virus in monkey monocytes.

Monkeys are presently the only available laboratory model for dengue infection, e.g. they exhibit viremia and antibody production but no overt sign of disease. The parent dengue-2 virus produces viremia and specific antibodies while the small plaque dengue-2 (PR-159) virus causes an antibody response without producing detectable viremia. We wanted to know if replication in monocytes in vitro correlated with the presence of viremia.

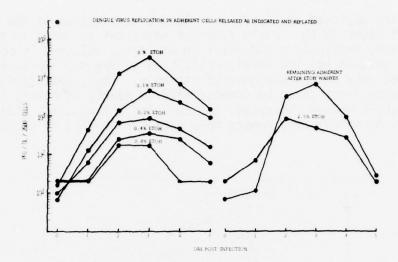


FIGURE 12. Replication of dengue 2 virus in monocytes with varying adherence properties.

Monocytes were obtained from normal, dengue-2 immune, and dengue-3 immune monkeys (two each). The originally adherent cells rather than secondary adherent cells were used since monkey monocytes were too fragile to withstand the scraping and replating used to obtain the secondary adherent cells. In addition, monkey monocytes were not strongly adherent; we lost over half of them during the process of infection and removal of the inoculum.

Attempts to demonstrate either parent or small plaque virus replication in the monkey adherent cells cultured in normal medium were not successful. We had thought that residually attached immunoglobulin on the monocytes from the dengue immune monkeys might facilitate infection and replication via immune enhancement, at least with the parent virus. Monocytes from the six monkeys were also cultured in medium containing dengue-2 antibody; no small plaque virus replication occurred at all, and only a few PFU of parent virus was obtained from the monocytes from one of the six monkeys. These results are quite different from the virus production observed in human monocytes.

Since we observed in the preceding section that weakly adherent human monocytes may replicate dengue virus to higher titer than the strongly adherent cells, we attempted infection with weakly adherent cells. Following removal of the nonadherent cells, the dishes were washed gently, and the remaining cells were removed with the vigorous agitation and pipetting. These cells were infected and maintained as a quasisuspension culture (agitation twice a day).

The results are depicted in Figure 13. Again, replication of the parent virus did not occur in the weakly adherent monocytes cultured in normal medium; however, replication did occur in monocytes cultured in dengue-2 antibody. A delayed and low level replication of the small plaque virus occurred only in the presence of antibody. Thus, replication via immune enhancement could be demonstrated in weakly adherent, but not strongly adherent monkey monocytes, and explains the problems that investigators have had using monkey monocytes for studies of dengue replication.

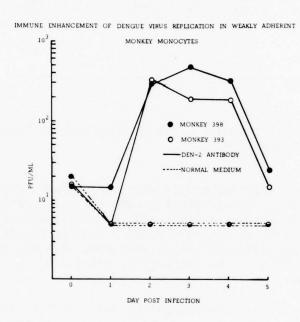


FIGURE 13. Dengue virus replication in weakly adherent monocytes from normal monkeys. Replication occurs only in the presence of non-neutralizing. antibody.

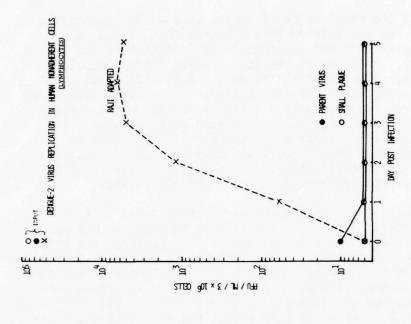
## B. Dengue infection of human lymphocytes

The study of the replication of dengue viruses in lymphocytes (nonadherent cells) was not pursued to the extent that it was in monocytes since lymphocytes were not susceptible to infection during the first day in culture. As shown in previous studies (Theofilopoulos et al., 1976) the Raji adapted strain of dengue 2 (16681) virus would only replicate in lymphocytes that were cultured for several days after isolation. This virus replicated to a higher titer and was detected earlier in the growth curve when the lymphocytes were cultured for several days with pokeweed mitogen or phytohemagglutinin prior to infection, suggesting that the "T" cell subpopulation of lymphocytes might be supporting virus replication. However, dengue was found to replicate only in lymphocytes having "B" cell characteristics.

The replication of Raji adapted dengue virus in 3-day cultured lymphocytes may represent a laboratory artifact; however, we evaluated the ability of the PR-159 strain of dengue-2 virus, passaged six times in certified primary monkey kidney cells (GMK-6), to replicate in lymphocytes and compared it to the Raji adapted virus in aliquots of the same cells. Infection was accomplished by resuspending 2 x 107 nonadherent cells in 1 ml of inoculum containing 2 x 10<sup>5</sup> PFU (MOI = 0.01). Following incubation for 1½ to 2 hours at 35 C in a CO2 incubator with occasional agitation, the cells were centrifuged from the inoculum at 350 x g for 7 minutes at 4°C. The residual virus inoculum was removed from the cells by repeated (three times) resuspension in 12 ml of blood diluent and centrifugation. The fourth resuspension was in 12 ml of growth medium which was dispensed in 2 ml aliquots into small plastic petri dishes (Falcon 3001 -  $35 \times 10$  mm). The remainder was frozen as a 0 time sample. Daily samples were obtained for 5 days by freezing the contents of 1 dish from each donor.

The growth curves in monocytes from two donors are presented in Figure 14. Only the Raji adapted dengue virus replicated in the lymphocytes. The PR-159 GMK-6, virus strain did not replicate in lymphocytes even at a 10-fold greater MOI. Thus, the dengue-2 GMK-6 virus could be differentiated from a laboratory strain that was adapted to transformed cells.

The GMK-6 virus was used as the wild parent virus from which the attenuated small plaque forming vaccine candidate was isolated (1972-1976 Annual Reports). Since lymphocytes were not susceptible to infection with the parent virus, we carried out several more experiments to determine if the small plaque Dengue-2 PR159 vaccine virus candidate had acquired the ability to replicate in lymphocytes. A representative experiment, shown in Figure 15, indicated that neither the parent nor the small plaque virus replicated in lymphocytes, while the Raji- adapted virus, used as a positive replication control, replicated relatively well.



DENGUE-2 VIRUS REPLICATION IN HUMAN LYMPHOCYTES

- RAJI ADAPTED VIRUS ---- GMK-6 VIRUS

O DONOR - D.P. ODONOR - L.A.

Replication of Raji adapted dengue virus in nonadherent cells from 2 two donors. Dengue-2 virus passaged in primary monkey kidney cells did not replicate. FIGURE 14.

FIGURE 15. Evaluation of the small plaque vaccine candidate to replicate in lymphocytes as compared to the parent dengue-2 virus and a virus adapted to a lymphoblastoid cell like (Raji-adapted).

roc10 PFU/ML

TIME IN DAYS

Finally, we determined if the parent and small plaque viruses would replicate in lymphocytes as in monocytes when antibody was included in the culture medium. Again, these two viruses did not replicate; however, the Raji adapted dengue-2 virus which served as a positive replication control for the lymphocytes was inhibited by the same antiserum that enhanced virus production in monocytes as described above. Almost identical results was obtained with lymphocytes from separate donors studied one month apart (Figure 16); quantities of Raji adapted virus in the normal culture medium increased substantially following the initial detection of new virus progency two days post infection. New virus progency in the culture medium containing heterologous dengue-3 antibody was also first detected two days post infection, but the quantities were 10-fold less, and decreased over 100-fold after the third day post infection.

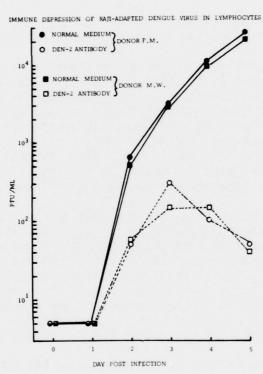


FIGURE 16. Effect of non-neutralizing antibody on the replication of Raji adapted dengue virus in lymphocytes.

This represents another biological distinction between the non-adherent (lymphocytes) and adherent (monocyte) white cells and adds yet another parameter attesting to the validity of the method used in this report to study replication in the two types of cells: Monocytes are essentially immediately infectable and can produce greater quantities of virus in the presence of antibody; lymphocytes have to be cultured before they become susceptible to infection and virus production is depressed in the presence of antibody. This base-line data was extended further by investigators in the SEATO laboratory when they showed that virus isolation was accomplished from monocytes (adherent cells) recovered from dengue patients rather than the non-adherent cells containing the various population of lymphocytes. (LTC R.M. Scott, personal communication)

C. The effect of noninfectious viral antigens on the sensitivity of plaque reduction neutralization tests for dengue antibody.

Identification of virus strains of subtypes is ultimately accomplished by neutralization of their infectivity with specific reference antisera. Investigators from many laboratories must collaborate in the identification of arthropod-transmitted viruses isolated from materials collected in the field. Although different laboratories may use the same reference antisera for virus identification, variations may be found in antibody titer from lab to lab. These titers are important when cross neutralization tests are evaluated for revealing previously unrecognized viruses, or identifying new strains of known viruses.

Infected suckling mouse brain is often used as the source of virus for a neutralization test; one laboratory may use a low dilution of the brain homogenate while another laboratory may use a relatively high dilution to obtain a certain number of infectious units to be neutralized by the antiserum. The following study was designed to determine if excess non-infectious antigen in low dilutions of brain homogenate could compete for antibody in a plaque reduction neutralization test (PRNT) and be measured by a reduced titer of neutralizing antibody. Dengue-2 virus (New Guinea 'C') was chosen for the experiments.

Several approaches were carried out to examine the competing relationships between infectious and non-infectious antigen: 1) using the infected mouse brain in various stages of purification as the source of virus for a PRNT; 2) progressive heat inactivation of a mouse brain homogenate such that gradually lower dilutions of the brain homogenate had to be used to obtain the test dose of 100 plaque forming units (PFU); 3) complete ultraviolet inactivation of virus concentrated by pelleting in the ultracentrifuge and adding it to 100 PFU prior to mixing with dilutions of the antisera.

1. Virus from various stages of purification for neutralization tests.

Physical and chemical treatment of infected mouse brain homogenates can be selected to decrease the quantity of host tissue as well as the excess viral specified antigens not incorporated into mature virions. The following samples (also listed in Table 2 from left to right) in order of treatment served as a source of virus for the PRNT: a) Crude supernate (6,000 x g supernate of a 20 percent infected mouse brain suspension in Earles balanced salt solution); b) Crude pellet  $(6,000 \times g \text{ sediment from a})$ ; c) Protamine supernate  $(6,\overline{000} \times g \text{ supernate})$ after 1 mg/ml protamine sulfate mixed for 45 minutes with crude supernate); d) <u>Protamine pellet</u> (6,000 x g sediment from c); e) <u>Ultracentrifuge</u> <u>supernate</u> (residual virus after sedimenting it in the ultracentrifuge); f) Ultracentrifuge pellet (sedimented virus concentrated about 50-fold); g) Virions (purified particles obtained as a rapidly sedimenting hemagglutinin (RHA) from sucrose gradients (Smith et.al., 1970; Brandt et.al, 1970); h) Midgradient peak infectivity (trailing virions plus virion fragments from a sucrose gradient); i) Trailing peak infectivity (trailing virions in a sizeable peak of non-infectious slow-sedimenting hemagglutinin (SHA). Each of these samples was adjusted to give a final concentration of 20 percent fetal bovine serum in order to provide a protective protein matrix during freezing and thawing necessitated by storage at -70°C. Aliquots were titrated by plaque assay in LLC-MK2 cells (Eckels et al, 1976) and appropriate dilutions were calculated to obtain a plaque count of about 100 PFU per test. The duluted virus was mixed with serial two-fold and sometimes intermediate dilutions of reference antibody prepared in mouse ascitic fluid by standard procedures (Brandt et al, 1967; Chiewsilp and McCown, 1972). The accuracy of virusantibody mixtures were sometimes checked by the addition of radioisotope. The mixtures were incubated for 30 minutes at room temperature and then inoculated onto LLC-MK2 cell monolayers to measure residual virus by plaque assay. The titer of the antiserum when tested against each of the infectious antigen samples is shown in Table 2 and represents that dilution which neutralized 50 percent of the plaque counts in that particular antigen preparation.

It can be seen in Table 2, left side, that each step of antigen purification resulted in the detection of a higher titer of antibody in the antiserum. It would appear that the use of purified virions would enhance the titer of antiserum considerably, but storage of the virions under our conditions resulted in much lower antibody titers in repeated tests; optimum storage conditions are yet to be determined. The right side of Table 2 lists the titers of the antiserum when it was tested against each of the materials that are ordinarily discarded following each step of purification of the virus. It can be seen that the antiserum titers are 2-3 fold lower than with the corresponding materials on the left side of the table, suggesting that excess non-infectious antigen on the right may be competing for the antibodies. It can also be seen that the antiserum titers gradually increased when testing it against the

discarded material from each successive step of purification. The lowest titer was obtained with the crude pellet, which is the material containing all of the particulate matter from the mouse brain homogenate (i.e. cell membranes containing viral antigen and proportionately much less infectious virus). It would appear that the particulate material was the most effective at combining with the antibody and left the least unbound antibody to neutralize the infectious virus trapped in the crude pellet.

TABLE 2. Effect of different stages of purification of dengue-2 virus on the titer of a reference antibody preparation.

Material for further Purification	Antibody titer	Material ordinarily discarded	Antibody titer
Crude supernate	1:1300	Crude pellet	1:640
Protamine supernate	1:1750	Protamine pellet	1:860
Ultracentrifuge pellet	1:2560	Ultracentrifuge supernate	1.940
Virions	1:7000	Trailing infectivity	1:1600
		Mid-peak infectivity	1:2560

The protamine pellet contains the soluble brain material which also has noninfectious antigen associated with it plus infectious virus trapped in the protamine precipitate. The antiserum has a slight but insignificantly higher titer when tested against the protamine pellet suggesting that it might be "cleaner" than the crude pellet. The ultracentrifuge supernate would appear even "cleaner" than the crude pellet since the antiserum titer is still higher. The ultracentifuge supernate is free of cell membranes and considerable soluble host components containing noninfectious antigen, but it still contains surface subunits and larger size fragments of the virion which can combine with antibody leaving it unavailable to neutralize the infectious virus. The material labeled "trailing infectivity" represents some purified virus mixed with a considerable quantity of excess non-infectious virion surface antigen that appear as ring forms or "doughnuts" under the electron microscope. This material appeared to inhibit the neutralization reaction but not as much as the other discarded materials. The mid-peak infectivity contained some fragment of virions mixed in with the infectious virions, and this material inhibited the neutralization reaction the least. The antibody titer obtained with the mid-peak infectivity sample should compare favorably with the antibody titer obtained against the

ultracentrifuge pellet. Both preparations contain non-infectious and infectious antigen in about the same proportions and, indeed, the reference antibody preparation exhibited a titer of 1:2560 against each. It would appear that partial purification of virus preparations improves the sensitivity of the neutralization test, probably due to the removal of excess virus specific but non-infectious antigen which competes for neutralizing antibody.

2. Heat inactivation as a method to increase the proportion of non-infectious to infectious antigen for neutralization studies. The effect of increasing noninfectious antigen with a constant number of plaque forming units in a neutralization test was approached by gentle heat inactivation of the infected mouse brain suspension; the heated samples were titered, adjusted to 50-100 plaque-forming units, and mixed with dilutions of reference antibody. Heat inactivation of the infected mouse brain suspension was carried out at two temperatures, 37°C and 40°C (Figure 17). It can be seen that heating at 40°C reduced the number of plaque forming units in a convenient time interval (about 7 hours). Higher temperatures caused a precipitous and unpredictable decline of infectivity. The lower temperature of 37°C resulted in delayed and uneven inactivation.

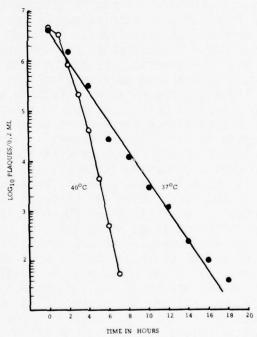


FIGURE 17. Residual dengue-2 virus in infected suckling mouse brain following incubation at 370C (closed circles) and 400C (open circles). The virus was assayed by plaque formation in LLC-MK2 cell culture.

The unheated infected mouse brain suspension (seed) was diluted 1:40,000, giving an average of 105 plaques for use in the neutralization test; the antibody titer obtained with this antiben preparation was 1:1600. When the dengue seed was heated at 40°C for 7 hours, we were able to use it undiluted for an average plaque count of only 26. This should have produced a more sensitive neutralization test since there were fewer plaques to neutralize, but the titer of the antibody was only 1:500, about 3-fold lower than when the antiserum was tested against diluted and unheated seed material. When the dengue seed was heated for 5 hours, we diluted it 1:10 to obtain a plaque count of 59. This dilution resulted in the reference antibody having a higher titer of 1:1200, indicating that a substantial amount of excess non-infectious antigen (as when the undiluted seed was used) must be present to significantly block a neutralization reaction. Some indication of how much noninfectious antigen may be required to block a neutralization test is given in the next section.

3. Addition of ultraviolet inactivated hemagglutinins to dengue virus prior to the neutralization test.

An experiment to determine if neutralization titers could be blocked in excess of four-fold was carried out by inactivating pelleted hemagglutinins with ultraviolet (UV) light. Pelleted hemagglutinins isolated from infected mouse brain contain approximately equal proportions of infectious and non-infectious hemagglutinins separable as rapid and slow sedimenting peaks on sucrose gradients (Figure 10, 1969 Annual Report). UV light was chosen as an effective method of cold sterilization, about 3 minutes being required to reduce the plaque titer of a sucrose purified virion preparation about 10,000-fold using 2000 erg/cm<sup>2</sup>/sec (Figure 13, 1968 Annual Report). Since pelleted hemagglutinins represented an unpurified concentrated virus preparation also contining UV absorbing contaminating proteins, a high power UV lamp, having an intensity in excess of that directly measurable by present day meters, was chosen and first shown to reduce the infectivity of a sucrose purified dengue virus preparation 100,000 fold in less than 30 seconds. The unpurified pelleted hemagglutinins were exposed to the high power UV light for 4 minutes, which completely inactivated 10<sup>9</sup> infectious units. The effect of this material on a neutralization test was tested in two ways: a) it was mixed with an equal volume of dengue seed diluted to contain 200 plaques/0.1 ml, such that after addition of an equal volume of the antibody dilutions, 100 plaques/0.2 ml would be available for inoculation on LLC-MK2 cells (as indicated by control dilutions with normal serum); b) a half volume of the UV inactivated hemagglutinins was added to the antibody dilutions first, incubated for 2 hours, then the diluted dengue seed was added. In both tests, the added UV inactivated hemagglutinins reduced the plaque count in the controls approximately 50 percent, clearly interfering with the ability of the antibody to neutralize dengue virus. Antibody dilutions commencing at 1:300 were unable to reduce the observed plaque count in the UV hemagglutinin-seed mixtures, whereas a 1:1500 dilution of the antibody reduced the plaque count of the dengue seed material alone by 50 percent. A substantial amount of additional non-infectious antigen was used here to reduce an antiserum titer in excess of five-fold.

In section 2, increasing the amount of non-infectious antigen in a seed preparation by heat-inactivating most of the infectivity that was already there reduced the antibody titer by 3-fold, and partially purifying the virus as shown in section 1 increased the titer of the antibody by 2 to 3-fold. Since we had to add additional antigen to the system to reduce the antibody titer in excess of 5-fold, it would appear that variations of this extent should be resolved between laboratories on a technical basis. These observations can only be applied to virus obtained from infected suckling mouse brain. Our prior experience with infected cell culture media was quite different (1969 Annual Report). Dengue virus released into the media from cells in culture is generally now accompained by a release of an equivalent amount of non-infectious antigen. Only when the cells are harvested and disrupted can quantities of non-infectious antigen be recovered. Thus, as long as no cytopathology is present, virus in cell culture fluids is much cleaner than virus in infected mouse brain homogenates. Pelleting the virus from cell culture fluids and using it in a neutralization test reduced, rather than increased, the titer of a reference antibody, probably due to a combination of a) neutralized aggregates that disaggregated on inoculation into the assay system and b) simultaneous concentration of competing non-infectious antigen. Pelleting the virus from infected mouse brain on the other hand, left behind enough of the contaminating non-infectious antigen released by the disrupted cells that the antiserum titer was increased. While other factors affect the titer of reference antibody preparations, (i.e. inherent variations in 2-fold dilutions, fresh isolates of a given dengue strain tend to elicit lower reference antibody titers than higher passages of the same strain), the source of the virus seed material should be considered prior to experimental manipulation. A diluent that can prevent aggregation of highly purified virus particles, as well as protect the purified particles during frozen storage, would greatly assist antigenic analysis of dengue viruses.

# D. Replication of dengue virus in rabbit kidney cells

Recent information obtained from a collaborative study with the Scripps Institute indicated that dengue virus replicated to moderate titers in rabbit endothelial cells (REC); Raji adapted dengue 2 virus replicated to titers at least  $10^4$  PFU/ml and mouse brain passaged virus replicated to titers of at least  $10^2$  PFU/ml. The pathophysiology of dengue hemorrhagic fever may evolve from both direct damage to endothelial cells by infectious virus and the release of complement activators by these cells as a result of infection.

It has previously been observed in this laboratory that dengue virus does not replicate in rabbit kidney cells (RKC) but does induce the synthesis of the soluble complement fixing (SCF) antigen, presumptive evidence of an abortive infection. Endothelial and kidney cells are both of mesodermal origin and might be expected to respond similarly to dengue infection. Therefore, additional studies were done to establish whether or not dengue does cause an abortive infection in RKC.

Kidney cell cultures from 2-3 week old rabbits were obtained from Flow Laboratories, Rockville, MD, or prepared from fresh rabbit kidneys at WRAIR after trypsinizing for two hours or overnight. (Rovozzo et al, 1973). The source of cells did not affect the results. RKC were grown in 25 cm<sup>2</sup> plastic flasks in Earl's minimal essential medium supplemented with 2% fetal bovine serum, penicillin and streptomycin. REC used between passages 20 and 45 were derived from the vena cava of a normal animal and maintained in RPMI 1640 containing 20% heat inactivated fetal calf serum. A mouse brain seed of dengue 2 (New Guinea Strain) was adsorbed to confluent monolayers of RKC or subconfluent REC for 1-2 hours at 36°C. Virus was then removed. REC was washed three times; RKC were washed six times with maintenance media. Each wash involved the addition of medium vigorous shaking with subsequent incubation for 5 minutes and aspiration of the wash. Monolayers were fed with medium and incubated at 36°C. At appropriate times, the supernate from duplicate flasks was combined and stored at -70°C until tested by plaque assay. RKC were washed twice, covered with a minimal amount of TBS and stored at -70°C until analysis of SCF antigen was done. The plaque assay was performed in LLC-MK2 as previously described (Eckels et al, 1977). Determination of SCF antigen has been also described (Brandt, et al, 1970). CF antigen was found within the RKC; we have not yet determined if it was synthesized in the absence of mature progency virus. We were surprised to find that mouse brain dengue virus adhered tightly to RKC but not to REC or monkey kidney cells.

Viral growth curves in both REC and RKC are seen in Figure 18. Dengue replicated to low titer with peak production in REC at 48 hours whereas in RKC substantial amounts of dengue were present in the 2 hour sample with subsequent steady decline. No cytopathic effect was seen in RKC. The preliminary studies do not confirm the previous observation that dengue virus does not replicate in RKC. The low level of replication initiated by the mouse brain seed in the rabbit endothelial cells could have occurred also in the kidney cells but was hidden by the unusual high binding of the dengue inoculum and subsequent slow elution over several days. Further work is underway to clarify the observations by using dengue virus from cell cultures (including Raji cells) and by varying multiplicities of infection.

The extremely tight initial binding of dengue mouse brain seed virus to the RKC was not expected and is not found with dengue in other cell systems including the REC. This attachment could lead to the belief that dengue replicates in RKC if only a 24-hour sample was plaqued. Initial

binding with subsequent elution of infectious virus has also been observed for myxoviruses, picorna viruses and polyoma viruses. (Lowberg-Holm, and Philipson, 1974). The release of some viruses is due to the action of a receptor destroying enzyme on the virion, but for most others the mechanism is unclear and is dependent on the ionic strength, temperature, pH, and divalent cations of the medium. The role of these variables on the interaction of dengue and RKC has not been investigated. Binding and growth in RKC must be determined for Raji-adapted dengue virus which differs in lipid content from mouse brain virus. It may be that the higher and undoubtedly qualitatively different lipid content of mouse brain virus is responsible for the firm initial attachment. If the SCF antigen is produced in RKC when complete virus replication has been shown not to occur, then such an abortive infection may prove useful in analyzing intracellular controls of dengue replication.

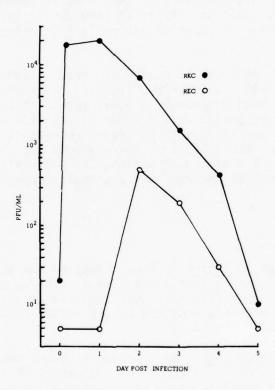


FIGURE 18. Binding of dengue infected mouse brain inoculum to rabbit kidney cells as contrasted to rabbit endothelial cells.

## E. An epidemic of dengue in Jamaica - 1977.

### 1. Background

In March and April 1977, an increasing number of febrile illnesses occurred in a garrison of young military men in Up Park Camp, Kingston, Jamaica. By mid-May, over 300 cases were observed. Dengue was suspected and confirmed by serological tests in the Department of Microbiology, University of the West Indies (UWI). Retrospectively, the onset of the earliest confirmed case in Kingston was 26 February 1977.

Ten acute sera were sent to the CDC San Juan Tropical Disease Laboratory for virus identification. Three sera were found to infect inoculated mosquitoes; the virus was identified as dengue type 1 by complement fixation. This was the first time dengue type 1 (Den-1) had been identified as the cause of naturally transmitted disease in the Western Hemisphere.

Although large epidemics of dengue occurred in Jamaica in 1963 (Griffiths et al, 1968) and 1968-1969 (Ventura and Hewitt, 1970) and sporadic cases have been diagnosed in other years, it soon became apparent that far more people were ill during the 1977 epidemic than at any previous time. In response to an urgent request, a coordinated approach to the study and control of the epidemic was developed among representatives of the Ministry of Health, The University of the West Indies and consultants to the Pan American Health Organization from the WRAIR.

#### 2. Methods

- a. <u>Virus isolation</u>. Virus isolation was accomplished by direct plaque assay in a continuous line of LLC-MK2 cell cultures. Virus isolates were identified by plaque reduction neutralization tests utilizing reference antisera provided by the Walter Reed Army Institute of Research (WRAIR).
- b. Antibody detection. Dengue antibody was detected by a microtiter modification of a standard hemagglutination inhibition test of paired sera using suckling mouse brain antigens to Den-1, 2, 3 and 4 produced by the SEATO Medical Research Laboratory, Bangkok, and provided by the WRAIR. In order to conserve antigens, sera were initially screened with DEN-1 or -2 antigens. Low titered or negative sera were tested with all four dengue antigens as needed. A four-fold rise in HI antibody titer or a "high-fixed titer" of greater or equal to 1:640 on at least one of two sera was accepted as presumptive evidence of a recent dengue infection.

Epidemiological studies were directed toward describing serologically confirmed cases; searching for cases complicated by hemorrhage, shock or death; following the progress of the epidemic throughout the island; and determination of the ratio of inapparent: apparent infections in adults.

- c. Description of the disease. Three approaches were used to describe the disease syndrome. Patients were examined in clinics while acutely ill and blood samples were drawn for virus isolation attempts. A retrospective review was made of the log books of four parish casualty centers by Dr. Carlos Sanmartin, PAHO consultant, to determine the age-specific presenting complaints of febrile patients. Lastly, a questionnaire survey combined with the collection of paried sera from industrial workers permitted the association of specific symptoms in adults to serological evidence of infection.
- d. <u>Case reports</u>. Cases of complicated dengue with hemorrhage, shock or death were sought and investigated by interviewing physicians and reviewing autopsy reports. Dengue infection was considered substantiated only if serologic evidence of recent infection was obtained and there was no other explanation for disease.

The progress of the epidemic was monitored through reports to the Ministry of Health from health centers in each parish. Twenty-two sentinel reporting stations, one in each parish (12) and 10 in metropolitan Kingston, were selected for their ability to provide regular weekly reports. Each station provided data on the weekly total patients with dengue and otherwise unexplained fevers. Every twentieth febrile patient was to have an acute and two week convalescent blood sample drawn for serological testing at U.W.I.

To estimate the scope of the epidemic, the age-specific incidence rates were determined for cases reported by the 10 sentinel stations in Kingston. To estimate the total number of adults infected, the inapparent: apparent infection ratio was estimated by surveying two industrial populations.

## 3. Identification of Dengue virus type 1

A virus isolated in LLC-MK2 from an acute serum collected in Kingston on 19 July was identified as DEN-1 12 days later by direct plaque assay in the presence of typing antisera. Five additional strains of virus obtained from Jamaicans and one strain from a resident of Louisiana who had recently returned from Jamaica have also been identified as Den-1 by plaque reduction neutralization test (Table 3).

TABLE 3. Identification of six dengue virus isolates by plaque reduction neutralization test.

Virus			Date Serum	Recipr	ocal Tite	er of New	it.Antibody
Strain	Source*	Specimen	Collec- ted	Den-1	Den-2	Den-3	Den-4
H13802	SJTDL	LLC-MK <sub>2</sub>	17 May	70	<10	11	<10
H13806	u	u	17 May	150	<10	<10	<10
14149**	11	serum	13 July	90	<10	<10	<10
CV 1515	UWI	u	12 July	88	<10	<10	<10
CV 1536	n	II .	13 July	100	<10	<10	<10
CV 1570	n	II .	14 July	100	<10	<10	<10
(Homologou	us titer)			(1200)	(1700)	(640)	(160)

<sup>\*</sup> SJTDL is San Juan Tropical Disease Laboratory; UWI is University of the West Indies.

#### 4. Description of the acute illness.

Table 4 lists the frequency of the most common symptoms of adults with serologically confirmed secondary dengue infections. These adults were employees interviewed by questionaire during a survey of two companies. The symptoms are similar to those described previously for patients with dengue fever.

A retrospective review of the casualty room log books at the Spanish Town Clinic suggested that there were age-specific differences in the relative frequency of presenting complaints. The presenting complaints of 1471 people from 1-7 May were compared to 1653 people seen from 15-21 June, at the peak of the epidemic. During the epidemic, fever was common in all age groups up to 59 years and was usually the only complaint in children under 5 years. Painful muscles, eyes and/or headache were common in all ages over 4 years and became the predominant symptoms after age 20 years. Headache was seen with increased frequency between 10 and 40 years and abdominal pain after age 40 years. Gastrointestinal

<sup>\*\*</sup> Serum obtained from a resident of Louisiana who recently returned from Jamaica.

and respiratory complaints were increased only in children under 10 years. The presenting complaints seen at three other casualty rooms at Port Antonio, Montego Bay and Mandeville were similar to those seen in Spanish Town.

TABLE 4. Frequency of specific symptoms in adults with confirmed secondary dengue infections.

	Company A (35)	Company B (69)	Total (104)	Percent
Back Pain	14	13	27	26
Headache	14	12	26	25
Feverishness	16	7	23	22
Loss of Appetite	15	6	21	20
Muscle pain	12	8	20	19
Eye pain	13	7	20	19
Leg pain	10	8	18	17
Abdominal pain	6	11	17	16
Nausea	10	2	12	12
Rash	5	2	7	7
Cough	5	2	7	7
Diarrhea	5	2	7	7
Unusual bleeding	2	1	3	3
Vomiting	2	0	2	2
Sorethroat	1	1	2	2

## 5. "Dengue" with complications

Personal observations revealed petechiae on the hard palates of some adults during the acute phase of disease, but few people reported any unusual bleeding and tourniquet tests were uniformly negative in all children and adults tested. A nonproductive cough was often observed during the second week of illness and many private physicians reported seeing a post-dengue depression or malaise in adults. No cases were found of dengue hemorrhagic fever or dengue shock syndrome in children.

There were several reports of "complicated dengue" in adults; patients with bleeding, shock and/or death following the onset of "typical" symptoms of dengue. None of the patients had virus isolation studies and none have been serologically confirmed. This does not mean that dengue with hemorrhage or death did not occur during the epidemic. The following case is representative.

A 30+ year old lady was interviewed in Nuttal Hospital on day 10 of her illness. Her initial complaints were fever, headache and leg pain followed by weakness and photophobia. On day 4, she developed hematemesis and melana. Her platelet count was 30,000, prothrombin time 13 sec/control 13.5 sec., partial thromboplastin time 47 sec/control 38 sec. and hemoglobin 8.0 gm%. Two days later, her platelet count was 75,000 and wbc 3600 with neutrophils 47%, lymphocytes 47%, monocytes 6% and eosinophils 2%. This lady had no previous history of GI hemorrhage but had been taking a great many different medications for weight reduction. It is possible that she had a previously undiagnosed GI disorder, such as peptic ulcer disease, which became manifest with bleeding due to thrombocytopenia secondary to a dengue infection.

## 6. Progression of the epidemic.

Virtually all reports of dengue-like illness prior to 1 May came from metropolitan Kingston. Reports from the sentinel stations, however, showed that the number of cases from other parishes was similar to that in the capital (Figure 19). The geographical distribution of cases showed widespread disease by 25 June. By 13 August, over 4200 cases had been reported by the 10 sentinel stations in Kingston and over 6600 from the other 12 parishes (Figure 20). Serological confirmation of dengue infections has been made for 8 of 9 parishes that submitted paired serum samples (Table 5). It is generally accepted that sentinel stations reported only a small fraction of the cases seen in each parish.

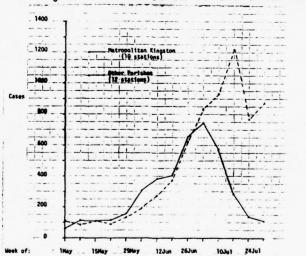


FIGURE 19. Cases of Dengue-like illness and PUO reported by sentinel stations.

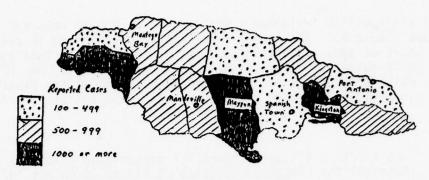


FIGURE 20. Cases of dengue-like illness in Jamaica reported by sentinel stations from 1 May to 13 August 1977.

TABLE 5. Serological tests of dengue-like illness submitted by sentinel reporting stations. 1 May to 23 July, 1977

	Dongue	Ser	um Sample	s	Onset of
PARISH	Dengue like illnesses	No. Cases Tested	No. Pos.	Propor. Pos.	First Con- firmed Den- gue Case
Hanover	249	1	1	1.00	July 11
Westmoreland	647	2	0	0.00	
St. James	539	0			
Trelawny	390	0			
St. Elizabeth	186	4	1	0.25	July 20
Manchester	541	3	1	0.33	July 8
St. Ann	42	25	16	0.64	June 26
C1 aredon	1093	14	12	0.86	June 6
St. Catherine	291	4	3	0.75	July 15
St. Mary	452	0			
Portland Portland	321	3	1	0.33	July 15
St. Thomas	811	0			
Kingston - St. Andrew	3907	166	134	0.80	Feb 26

The age-specific incidence rates indicated disease occurred in all age groups (Table 6). The lowest incidence rate was found in people over 50 years of age, suggesting some partial immunity in that age group. The incidence rates are based on unconfirmed case reports from 10 sentinel stations which probably serve less than 10% of Metropolitan Kingston and are generally believed to greatly underestimate the true incidence disease.

TABLE 6. Age specific incidence rates for dengue-like illnesses in Metropolitan Kingston, Jamaica 1 May to 23 August 1977.

Age	Estimated Population	No. Cases	Incidence Rates
0 - 4	87,500	390	446
5 - 9	86,800	332	382
10 - 19	170,100	344	202
20 - 29	126,000	394	313
30 - 39	77,000	176	229
40 - 49	51,000	100	193
50 or more	100,800	93	92
All ages	700,000	1829	261

### 7. Inapparent dengue infections in adults.

The inapparent: apparent infection ratio was determined for two employee populations from different locations. Information was obtained by questionaire interviews and serological testing of paired serum samples, collected at four week intervals between 20 July and 18 August.

Company A was a large bottling company located on the western edge of Kingston. It had 1000 employees, over 90% of whom were male. Of 349 employees interviewed, all but one were male. The median age was 34 years (range 16-37 years) and the median length of employment was 7 years (range 1 week to 49 years).

Company B processed citrus fruit and was located in south central Jamaica. It had a total employment of 650 people, of whom 164 (30 males, 134 females) were sampled. The median age was 39 years (range 17-71 years) and the median length of employment was 15 years (range 6 months - 50 years).

Study subjects from Company A were strictly volunteers who were aware that they were being studied for dengue infections. Subjects from Company B were involuntarily included in the study as part of their routine processing for food handler's permits.

An employee was considered to have an inapparent dengue infection if he did not report having "dengue" by name in 1977 or did not report having fever or more than one other common symptom of dengue during the preceding 30 days. Fever alone or two or more of the following complaints would have been counted as symptomatic illness: headache, back pain, eye pain, muscle pain and leg pain.

Dengue-like illnesses were reported by 119/349 (34%) of the employees of Company A and 71/156 (43%) of Company B, but all were not confirmed by hemagglutination inhibition. The inapparent: apparent infection ratios were 1:2 for Company A and 1:1 for Company B. (Table 7) The difference between the ratios reflects the bias introduced by the sampling methods. The estimated frequency of serologically confirmable dengue infections for each company was 34% (Company A) and 65% (Company B). These estimates support the impression obtained by casual inquiry of residents of Kingston. Virtually, everyone questioned at random in Kingston reported at least one case of dengue-like illness in their family (average size 4 people) during the summer of 1977.

TABLE 7. Frequency of inapparent dengue infections in two companies\*

		Company A	1		Company	В
Antibody Response	Inap- parent Infec- tion	Clinical Disease	Approx. Ratio	Inap- parent Infec- tion	Clini- cal Disease	Approx Ratio
"High fixed titer"**	11	24	1:2	29	40	3:4
Four-fold rise	5	8	1:8	28	17	7:4
Combined	16	32	1:2	57	57	1:1

<sup>\*</sup>Results are based on tests of 96 employees of Company A and 156 employees of Company B.

<sup>\*\*</sup> HI titer of  $\geq$  1:640 to Den-1 or -2.

Based on the frequency of infections found in Companies A and B, the estimated number of Jamaican adults of employable age who have had dengue in 1977 is 300,000 to 582,000. (Table 8)

TABLE 8. Estimated total dengue infections.

Study	Companies:		Α	В
1.	Total employees:	(A)	1000	650
2.	Est. Total 1977 dengue-like illness	ses:		
	employees reporting illness all employees interviewed	X A = (B)	341	277
3.	Est. confirmed dengue illnesses:			
	confirmed cases by HI X B all employees tested by HI	(C)	223	211
4.	Est. total dengue infections:			
	C + <u>inapparent infections</u> X C apparent infections	(D)	335	422
5.	Est. frequency of dengue infections:			
	total employees	X 100 = (E)	34%	65%
Jamai	ca Work Force	_		
6.	Est. total work force (Oct. 1976)*	= (F)	895500	
7.	Est. dengue infections:			
	ExF	=	304470	58207

<sup>\*</sup> Source: Daily Gleaner, 20 August 1977.

Company A had an outpatient clinic staffed by one physician and two nurses. The clinic was open for four hours, five days a week. All visitors were screened upon arrival and their major symptoms or type of illness was recorded in a log book. In order to determine the time period of greatest dengue illness in the company, a review was made of the log books for presenting symptoms of fever, pain and "dengue". As a control for the overall activity of the clinic, visits for trauma and minor surgery were recorded for comparison.

Dengue-like illness was seen in the Company A clinic from 9 May through the middle of August (Figure 21). Cases peaked from 13 June to 1 July about two weeks earlier than in Metropolitan Kingston.

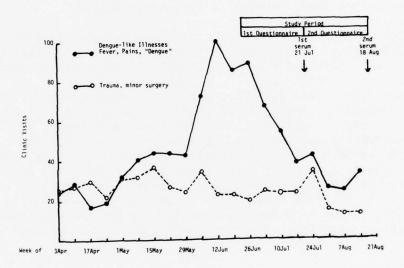


FIGURE 21. Weekly visits by employees to the clinic of Company A during a dengue epidemic, 1977.

#### 8. Discussion

Owing to an overall high susceptibility to the newly introduced dengue type I virus, the disease affected all age groups; the highest incidence rates were found in children and young adults. The relatively low rates seen in people over 50 years, however, is unexplained, but could be due to one or more reasons, including the following possibilities.

- a. Less severe illness in older people. The illness in older people was predominantly one of pain without fever and many cases may not have been recognized as dengue.
- b. Pre-existing immunity to dengue type I caused by infections many years ago in the Carribean as suggested by Rosen (1968). This hypothesis must assume that immunity is very long-lasting.
- c. Partial immunity conferred by multiple previous infections with other flaviviruses, such as dengue types 2 and 3 or St. Louis encephalitis virus, which could provide broadly reactive serum antibodies.

Since dengue virus type 1 has not been identified before in the Western Hemisphere, it must be assumed to have come to Jamaica from Africa or Asia. Several years ago, importation of new strains of dengue to the Carribean was predicted (Ehrenkranz et al, 1971) as a byproduct of the rapid means for international travel. This may not be the first time dengue type 1 has been in Jamaica, as suggested by the incidence rates for the older prople, and it probably will not be the last. However, in 1977, the dengue type 1 was introduced at a time when the very high level of general susceptibility and the availability of mosquito vectors were ideal for rapid, efficient and widespread virus transmission.

By the end of July, over 20 cases of dengue had been identified in the U.S. and others in Trinidad. Dengue I virus was isolated from at lease one person in Louisiana. All cases are considered to be imported from Jamaica and secondary transmission has not yet been observed.

Future outbreaks of dengue type 1 must be expected in Jamaica, but since so many people are now immune, it is unlikely there will be another epidemic of the magnitude reached in 1977.

F. The replication of California Encephalitis Virus in the presence of actinomycin-D, cordycepin and cycloheximide.

#### 1. Background

BFS-283 is the prototype strain of the California Encephalitis (CE) virus complex. The virus contains three major polypeptides and a

fourth minor peptide (1975 Annual Report). Electron microscopic studies showed that the initial cellular changes in infected cells occurred at the nuclear membrane level 15 min. after infection, as evidenced by vacuole formation involving the outer layer of the nuclear membrane (1976 Annual Report). Also, fluorescent-antibody studies showed that the first fluorescence was perinuclear, then spread throughout the cytoplasm of infected cells. These observations suggest that CE virus may have a nuclear phase in its replicative cycle or may be dependent on a nuclear product, in order to produce mature infectious virions. Also, we have shown that this virus is extremely sensitive to actinomycin-D in BHK21 cells at low concentration. The present investigation was undertaken to study three drugs that interupt viral protein synthesis at the RNA level. Although the drugs have multiple effects, they may be classified by the principle points of action: actinomycin-D (RNA synthesis), cordycepin (RNA-processing), and cycloheximide (RNA-translation).

A comparative study was done with CE and Sindbis viruses. Sindbis virus has a similar latent period to CE virus when infected at a similar MOI. Also, since Sindbis virus is known to be completely independent of the nucleus or nuclear products, it seemed to be the logical control virus to compare with CE virus.

#### 2. Methods

The following materials and methods were used.

- a. Cells. Baby Hamster Kidney cells (BHK21) were maintained as monolayer cultures in 32 oz prescription glass bottles, with reinforced Eagle minimal essential medium (E-MEM) containing 1% (v/v) fetal bovine serum (FBS). Secondary cultures for assay and inhibitory experiments were conducted in 60 mm falcon plastic petri dishes.
- b. <u>Virus</u>. Lyophilized BFS-283 CE virus was obtained from American Type Culture Collection (Rockville, MD) in the 24th mouse passage. Seed virus was prepared in 1-3 day-old mice as previously described (White, 1975).
- c. <u>Chemicals and Isotopes</u>. Mixtures of <sup>3</sup>H-L-Amino acids (<sup>3</sup>HAA) containing 15 amino acids (Specific activity 3.5 Ci/mmol), and <sup>3</sup>H-uridine (20 Ci/mmol) were purchased from New England Nuclear Corp., Boston, MA. Purified acrylamide and bisacrylamide were obtained from Bio-Rad Laboratories, Richmond, California. Actinomycin-D and cordycepin, were purchased from Sigma, St. Louis, MO.
- d. <u>Infection</u>. Cells were infected at a multiplicity of 10-50 with BFS-283 CE virus or Sindbis virus (AR 339) suspended in MEM containing 10% heat inactivated FBS.
  - e. Assay of Virus Infectivity. The infectivity titers

of BFS-283 and Sindbis viruses were determined by plaque assay on  $BHK_{21}$  cell monolayers

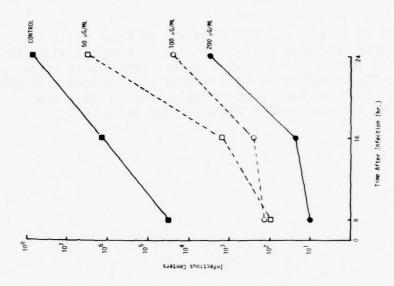
Pulse Labeling of Cells. BHK21/15 cell monolayers were washed twice with Hanks Balanced Salt Solution (HBSS) and infected with 10-50 PFU of virus per cell. Controls were cells mock infected with MEM containing 10% FBS. Incorporation of  $^3\mathrm{HAA}$  and  $^3\mathrm{H}$ -uridine into acid insoluble products was determined for various time intervals after infection beginning at 15 min. post infection. After a suitable pulse with 3HAA (20uCi/ml) in amino acid free medium or 3H-uridine (20 uCi/ml), net incorporation was determined by a method used by Scholtissek (1971) with minor modifications. The monolayers were washed twice with cold PBS to remove the residual radioactive medium. Further, unincorporated counts were removed by several washes with cold 6% TCA for the monolayers labeled with <sup>3</sup>H-uridine, and 10% TCA for those labeled with <sup>3</sup>HAA. The monolayers were then fixed by washing lx with ice cold ethanol followed one wash with ice cold methanol. Their integrity was ascertained by microscopic observation. For determination of the incorporated counts, the monolayers were hydrolyzed by the addition of 1 ml of 0.3 N NaOH to the petri plates. One tenth milliliter of this hydrolsate was mixed with 12 ml of a toluene based scintillation fluid and counted in a Packard Scintillation counter.

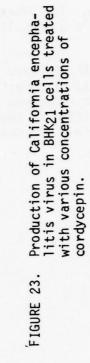
In cases where labeled proteins were to be analyzed by SDS-PAGE, the monolayers were extracted with 1 ml of 2% SDS after first washing them with cold PBS.

#### Results

Comparison of infectious virus production in CE and Sindbis virus infected cells.

When growth curves were performed in drug treated cells, Sindbis virus production was retarded by 200 ug/ml cordycepin at 8 and 16 hours after infection; however, by 24 hours after infection, cells treated with 100 and 200 ug/ml produced essentially the same amount of virus as the untreated control cultures. Further, cells treated with 50 ug/ml produced more virus than untreated cultures, thus showing a stimulatory effect on virus production. (Figure 22) In contrast, inhibition of infectious CE virus was directly proportional to the drug dose. The difference between the maximal dose (200 ug/ml) and untreated control cultures being that 20,000 times more infectious virus particles were produced in untreated cells (Figure 23).





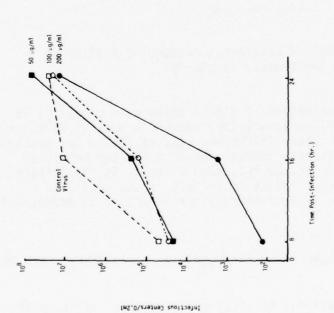


FIGURE 22. Production of Sindbis virus in BHK21 cells treated with various concentrates of cordycepin,

G. Inhibitory effects of BFS-283 CE virus on host-cell RNA and Protein Synthesis.

The effects on protein and RNA synthesis in  $BHK_{21}$  cells infected with BFS-283 are shown in Figure 24. Cells were infected at an multiplicity of infection (MOI) of 10-50 and virus was adsorbed to cells for 30 min. Removed cells washed twice in warm HBSS and replaced with MEM containing 2% FBS. Cultures harvested before and during this time (30 min) were washed twice with HBSS and dissolved in 1% SDS.

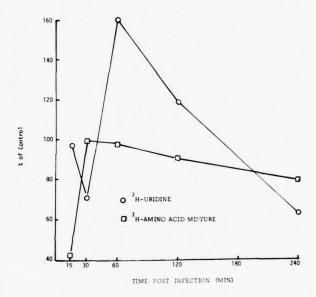


FIGURE 24. Inhibitory effect of California encephalitis virus on  ${\tt BHK}_{21}$  cell RNA and protein synthesis.

Infection caused an inhibition of 41% of amino acid uptake by 15 minutes after infection. Amino acid uptake reached a peak at 30-120 min after infection. At 15 min after infection, uridine uptake was the same in infected and control cultures; however, by 30 min after infection uridine metabolism was inhibited by 30%, then a rapid rise in infected cells occurred by 60 min after infection to 160% of the controls, followed by a rapid decline by four hours after infection to approximately 65% of the controls.

H. Effects of Actinomycin-D and cordycepin on normal  ${\rm BHK}_{21}$  cell RNA and protein synthesis.

Experiments were designed to study the effects of actinomycin-D and cordycepin on RNA synthesis in non-infected BHK $_{21}$  cells. The results

of these experiments are shown in Figures 25 and 26. These results indicate that both actinomycin-D and cordycepin cause immediate inhibition of RNA synthesis as measured by the inhibition of uridine uptake in BHK $_{21}$  cells. By 2 hours after treatment with actinomycin-D, uridine uptake was inhibited by more than 70%; however, at very low concentration (.002 ug/ml) the inhibitory effects diminished to the level of the control by 7 hours. Cordycepin treated cells inhibited uridine uptake to approximately the same extent as actinomycin-D in 2 hours, and increased to nearly 80% by 8 hours.

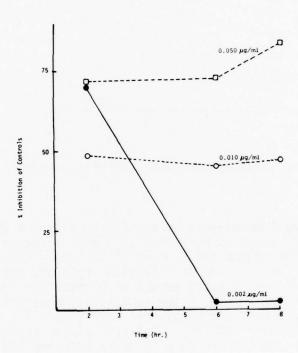


FIGURE 25. Effect of actinomycin-D on RNA synthesis in BHK-21 cells.

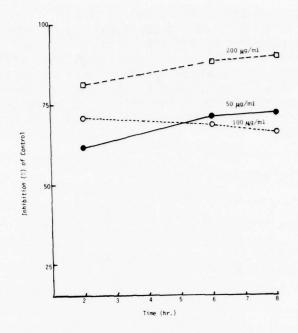


FIGURE 26. Effect of cordycepin on RNA synthesis in BHK-21 cells.

Briefly BHK $_{21}$  cell monolayers were infected with CEV BFS-283 at an MOI of 5-50. Control cultures were similarly mock infected. Care was taken to treat the two groups the same so as to keep manipulation artifacts to a minimum. At the time of infection replicate petri plates from both the control and infected groups were given the drug cordycepin at a concentration of 25 ug/ml. After an incubation period of 30 min to allow for viral attachment, the monolayers were washed 3 x with HBSS and then refed with E-MEM with 10% FBS. Replicate plates from both the control and infected groups were refed with media supplemented with cordycepin (25 ug/ml). All cultures were then pulsed with  $^3$ H-uridine (20 uCi/ml). at  $^1$ 5 min, 30 min, 1 hr and 2 hr post labeling, cultures from each group, i.e. (1) uninfected, cordycepin treated; (2) infected, cordycepin treated (3) infected, untreated; and uninfected and untreated, were assayed for incorporation of  $^3$ H-uridine into TCA precipitable products.

An almost linear curve described the rate of uridine uptake in the uninfected, non-drug treated cultures over the 2 hr labeling period. It has been our experience that this high rate of uridine incorporation is not maintained indefinitely, but tends to decline after 3.5 - 4.5 hr. This uptake probably reflects an increased metabolic rate in the cell due to re-feeding and certain manipulative stimuli.

In the cultures that were infected and/or cordycepin treated the initial rate of uridine uptake (over the first 15-30 min) was similar to that seen in the control cultures. However samplings after 30 min postpulse reveal that the rates of RNA synthesis decline rapidly in the infected and cordycepin treated cultures (Figure 27). We saw a slight rebound in the cultures that were infected but not drug treated at 1.5 - 2 hr post-pulse. In this case it is not known if the rise in RNA synthesis would have continued, since 2 hr represented the maximum labeling period in this experiment. Other investigators have shown that the synthesis of CEV RNA reaches a maximum at 4-6 hrs post infection. In the cordycepin treated cultures, both infected and uninfected, RNA synthesis has diminished to 10-15% of the control values by 2 hours.

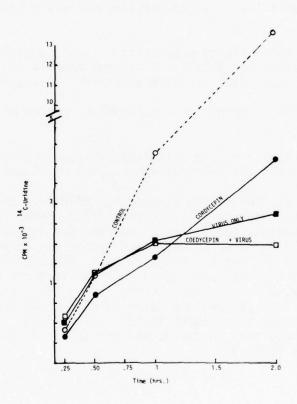


FIGURE 27. Kinetics of RNA synthesis in BHK-21 cells infected with California encephalitis virus and with cordycepin.

I. Viral isolations from human blood specimens collected in Brazil.

The specimens of whole blood processed for viral isolation were collected in Brazil by USAMRU-Belem between January 1974 and December 1976. The epidemiology program of USAMRU-Belem consisted of:

- (1) a prospective surveillance of a randomly selected sample of colonists living along the Transamazon Highway outside the towns of Maraba and Altamira. On the original visit to each colonist, the investigators recorded demographic and environmental information and collected blood for serology. Thereafter, the colonists were visited fortnightly, and for each episode of a febrile illness, the symptomatology was recorded and blood was drawn for virus isolation. Whether or not the person had reported an illness, blood for serology was obtained every six months.
- (2) a hospital surveillance program in which a sample of whole blood for viral isolation was obtained from each febrile patient admitted to the hospital in Maraba, together with a record of his symptoms.
- (3) investigation of epidemics, including epidemics of disease caused by arbovirus.

A detailed description of the epidemiology program of USAMRU-Belem may be found in the WRAIR Annual Reports for FY 75 and FY 76.

A total of 1635 specimens were shipped to WRAIR from the following study groups: Altamira Highway (246), Maraba (172), Maraba Hospital (1011), and epidemic investigations (206). Each specimen was inoculated into two tubes containing Vero cells to determine cytopathic effect (CPE) and into one litter of 3-5 day-old suckling mice (average litter size = 10). All suspected positives were passed into new Vero cells and mice and, if still positive, were put into LLC-MK2 flasks for a plaque titration. Viruses which form plaques are being identified by a standard procedure for plaque reduction neutralization test (PRNT). Details of all suspect isolates positive on the second passage in Vero cells or mice are presented in Table 9.

TABLE 9. Virus isolates recovered from human blood specimens collected along the Transamazon Highway.

Suspect isolate	Origin <sup>1</sup>	Date Collected	Vero Cells CPE	Mice	LLC-MK <sub>2</sub> plaque	Preliminary Identifica- tion <sup>2</sup>
1032	Marabá Hospital	Dec 74	+	+	+	Guaroa
4327	Marabá Hospital	May 75	+	+	+	Simbu Group
4129	Itupiranga	Jun 75	+	+	+	Simbu Group
41 86	Itupiranga	Jun 75	+	+	+	Simbu Group
10329	Marabá Hospital	Feb 76	+	+	+	To be done
2011	Marabá Hospital	Mar 75	+	+	+	Simbu Group
9556	Marabá Hospital	Jan 76	+	-	+	To be done
10344	Marabá Hospital	Feb 76	+	•	+	To be done
7530	Marabá Hospital	Sep 75	+	-	-	
11678	Altamira Highway	Apr 76	+		•	
8784	Marabá Hospital	Dec 75	-	+	•	
8759	Maraba Hospital	Dec 75	-	+	•	
4185	Itupiranga	Jun 75	-	+	+	To be done
8664	Marabá Hospital	Nov 75		+	•	
8740	Marabá Hospital	Nov 75	•	+	To be done	
4169	Itupiranga	Jun 75	1, 0	+	To be done	

 $<sup>^{1}</sup>$  Itupiranga is a small village near Maraba which experienced an epidemic of Oropouche fever in May and June 1975

<sup>&</sup>lt;sup>2</sup> Simbu group agents were identified by PRNT using antisera against 4327 and are probably Oropouche, but still require specific confirmation.

## II. Respiratory viruses

#### A. Adenovirus

 Acute Respiratory Disease (ARD) due to adenovirus and influenza in Basic Combat Trainees (BCT).

Fiscal year 1977 was characterized by perturbation in influenza and an unexpected calm in adenovirus ARD. Several remarkable events (or non-events) occurred with influenza:

- (1) Influenza A swine strains were not identified in the United States.
- (2) The occurrence of Guillan-Barre syndrome is less than 1 in 100,000 persons immunized with inactivated influenza A swine vaccine prompted a moratorium on influenza immunization in the military from 17 Dec 76 through 15 Feb 77. After mid-February 1977, only basic combat trainees received influenza immunizations until 1 May 1977 at one-half the standard dose of vaccine (which previously was 400 CCA units of influenza A/New Jersey/76 and A/Victoria/75 antigens and 500 CCA units of B/Hong Kong/72).
- (3) Influenza B caused illness at most BCT posts in January and February and was associated with peak ARD rates greater than 2.5/100 at Forts Bliss, Dix, Jackson, Knox and Wood.
- (4) A later wave of influenza A/Victoria/1975 occurred in February and March at several posts and was associated with rates above 2.5/100/week at Fort Dix. After influenza immunization of trainees was stopped on 1 May 1977, strains of influenza A/Victoria was isolated sporadically through June at several posts, but did not result in elevated ARD rates.

Isolates of adenovirus Type 4 and 7 were uncommon in BCTs in FY 1977. Except for a small outbreak of adenovirus Type 7 ARD at Ft. Knox in October (immunization with adenovirus Type 4 and 7 vaccines was delayed inadvertently until the week ending 23 October at this post), ARD caused by these types was insignificant. Both the Type 4 vaccine lots (Lot 07101 titering 4.9 log10 TCID50 per tablet used until March and Lot 07401 titering 5.1 log10 TCID50 per tablet used thereafter) and the Type 7 vaccine lots (Lot 07201 titering 6.2 log10 TCID50 per tablet used until March and Lot 07501 titering 6.1 log10 TCID50 used thereafter) effectively suppressed adenovirus type 4 and 7 ARD.

Adenovirus Type 21 was prevalent at all seven training posts throughout the year, but was not associated with sustained ARD rates above 2.0/100/week in the absence of influenza. The proportion of trainees hospitalized with ARD who yielded isolates of adenovirus Type 21 from the nasopharynx during the winter season rarely exceeded 35%, a proportion much less than the 40-60% previously encountered in outbreaks of Type 4 and

and Type 7 ARD. Rates of adenovirus Type 21 ARD observed were lower than anticipated. Indeed, Type 21 ARD disease rates observed were so minimal that it is questionable whether routine immunization of trainees with the live adenovirus Type 21 vaccine is required at present.

The following is a summary of respiratory disease patterns at the seven Army BCT posts under surveillance:

- a. Fort Bliss. Trainees were immunized with Type 4 and 7 vaccines from October through April. Only eleven strains of Type 4 (and no strains of Type 7) were isolated from the 414 (2.7%) ARD hospitalizations sampled during these months. A peak ARD rate of 1.3/100/week in February was associated with influenza B. Although adenovirus Type 21 was isolated from trainees throughout the 15 month fiscal year, ARD rates were otherwise below 1.0/100/week.
- b. Fort Dix. Type 4 and 7 vaccines were used from 1 September 1976 until 1 May 1977; during this period 25 isolates of Type 4 and 5 isolates of Type 7 were made from 1,412 trainees sampled (2.1%), and 231 strains of adenovirus Type 21 (16.4%) were made. The ARD rate peaked at 3.25 in February associated with seroconversions to influenza B in 56% and influenza A in 17% of the 149 hospitalizations sampled. Rates were below 2.0/100/week otherwise.
- c. Fort Gordon. Adenovirus vaccines were used from 1 October 1976 until 1 May 1977; Type 4 adenovirus was isolated from but 1 of 165 hospitalized trainees sampled (0.6%) while Type 21 was isolated from 21 trainees (12.7%) during these months. Median weekly ARD rates never exceeded 1.5/100/week in the 15 months of survey.
- d. <u>Fort Jackson</u>. Adenovirus Type 4 and 7 vaccines were used from 1 October 1976 until 1 May 1977. No isolates of Type 4 or Type 7 were obtained during these months, while Type 21 was isolated from 37 of the 303 hospitalized trainees sampled (12.2%). Median weekly ARD rates in the months of February and March 1977 were 2.7 and 2.1 in association with influenza A and B; rates in the other 13 months of this reporting period were consistently below 1.5/100/week.
- e. Fort Knox. Adenovirus Type 4 and 7 vaccines were used from 23 October 1976 until 1 May 1977. The late introduction of adenovirus vaccine was in part responsible for an October 1976 outbreak of adenovirus Type 7 ARD which is shown in greater detail in Table 10. During this outbreak, the proportion of hospitalized trainees with isolates of adenovirus Type 21 remained relatively constant (between 9-32%). Beginning the week ending 9 October, adenovirus Type 7 was isolated and the proportion of trainees with this type increased from 2% to 53% the week ending 23 October (after which immunization with Types 4 and 7 vaccine was begun). The increase in Type 7 activity paralleled an increase in ARD rates which peaked at 3.3/100/week during the week ending 6 November; thereafter both the ARD rate and proportion of Type 7 isolates declined.

This experience reemphasizes that adenovirus Type 7 can yet cause epidemics of ARD in unimmunized trainees and emphasizes the relative inability of adenovirus Type 21 to produce epidemic disease in the same environment.

TABLE 10. Acute respiratory disease due to adenovirus at Fort Knox, 1976.

Week	ARD rate/100/week	No. Sampled	% with i	solate of
			ADV-7	ADV -21
Oct 2	0.6	24	0	25%
9	0.8	47	2%	30%
16	1.4	28	11%	18%
23*	2.2	19	53%	32%
30	2.6	42	40%	21%
Nov 6	3.3	65	35%	25%
13	1.4	44	23%	14%
20	0.8	11	9%	9%
27	1.3	43	14%	19%
Dec 4	1.3	26	4%	27%
11	2.0	16	0	25%
18	0.6	45	0%	31%

<sup>\*</sup> Week ADV-4 and ADV-7 vaccines begun

After November, adenovirus Type 7 was isolated from only one of 805 hospitalized (0.1%) trainees sampled until immunization was terminated; during the same period adenovirus Type 21 was isolated from 149 (17.3%). Median weekly ARD rates were 3.1 and 2.9 in February and March 1977 associated with influenza A and B, but were below 1.5/100/week in other months.

- f. Fort Sill. Adenovirus Types 4 and 7 vaccines were used from 1 October to 1 May. One strain of adenovirus Type 7 was isolated from the 59 trainees sampled in this period, while adenovirus Type 21 was isolated from 5 (8%). The ARD rate remained less than 1.0/100/week throughout the 15 months of fiscal year 1977.
- g. <u>Fort Wood</u>. Adenovirus Type 4 and 7 vaccines were used through the 15 month period from July 1976 through September 1977. From October to May, one strain of Type 4 virus was isolated from 719 hospitalized trainees sampled, while Type 21 virus was isolated from 77 (10.8%). High ARD rates were experienced between December 1976 through March

h. Fort Wood. Adenovirus Type 4 and 7 vaccines were used through the 15 month period from July 1976 through September 1977. From October to May, one strain of Type 4 virus was isolated from 719 hospitalized trainees sampled, while Type 21 virus was isolated from 77 (10.8%). High ARD rates were experienced between December 1976 through March 1977 when median weekly rates were 3.4, 2.3, 4.0, and 2.9, respectively. The elevated rates were associated with influenza B from January through March when 7 of 32 (31%), 33 of 66 (50%), and 21 of 79 (27%) of hospitalized trainees, respectively, had serologic evidence of influenza B infection. Although adenovirus Type 21 persisted on post from October 1976 through August 1977, ARD rates after March were below 2.0/100/week.

## 2. Adenovirus Type 21 vaccine

Two field trials of the adenovirus Type 21 live virus oral vaccine were carried out in collaboration with the Division of Preventive Medicine and are described in detail elsewhere in this report.

a. Lackland AFB. In August 1976, a study of vaccine immunogenicity and safety was initiated in 476 Air Force volunteers at Lackland AFB, Texas. The study compared four groups who received combinations of adenovirus Type 4 (ADV-4), Type 7 (ADV-7), Type 21 (ADV-21) and/or placebo (P) vaccines. The four groups were ADV-4/7/21; ADV-4/7/P; ADV-21/P/P and P/P/P. Vaccine recipients were followed 21 days and evaluated for clinical illness and type specific neutralizing antibody responses.

The occurrence of illness was similar in all study groups. Neutralization tests which were performed in primary human embryonic kidney (HEK) cells by a standard technique (1970 Annual Report), showed no decrease in the immunogenicity of the ADV-4 vaccine when all three vaccines were administered together (Table 11). A decrease was observed, however, in the proportions of volunteers who seroconverted to ADV-7 and ADV-21 when they also received the ADV-4 vaccine.

b. Fort Dix, NJ. In November 1976, the first trial of ADV-21 vaccine efficacy was attempted at Ft. Dix, NJ. Although ARD rates were less than 2/100/week at the beginning of the study, all three types of adenoviruses were present at Ft. Dix, and an increase in disease rates was anticipated.

TABLE 11. Type specific neutralizing antibody responses of adenovirus vaccine recipients (Lackland Air Force Base, 1976).\*

	Adenovirus Type 4	s Type 4	Adenovir	Adenovirus Type 7	Adenoviru	Adenovirus Type 21
Vaccine	Initial	Three week	Initial	Three week	Initial	Three week
Study	titer <1:2	titer >1:4	titer <1:2	titer >1:4	titer <1:2	titer >1:4
group**	No.	No. (%)***	. NO.	No. (%)	No.	No. (%)
4/7/21	77	(6.77.9)	77	48 (62.3)	77	45 (58.4)
p/p/21	NT****	LN	ŢN	IN	59	41 (69.5)
4/7/p	92	56 (73.7)	92	(78.9)	LN	LN
d/d/d	78	0 (0.0)	49	2 (4.1)	88	2 (2.3)

Data provided by MAJ E. T. Takefuji, et al, Division of Preventive Medicine Adenovirus vaccines are indicated by number, placebo by p. Percent of number having an initial titer of <1:2.

NT - Sera were not tested because of the limited availability of human embryonic kidney cell cultures. \*\*\* \*\*\*\*

Two groups of 300 volunteers each were studied; one group received ADV 4/7/21 vaccines and the other received ADV 4/7/P. Each group received the vaccines within 72 hours of arrival at Ft. Dix and was followed throughout the total period of basic training. Vaccine safety, immunogenicity and efficacy were estimated by monitoring dispensary visits by study volunteers and admissions to the hospital. Virus isolation was attempted from throat swabs obtained from the volunteers admitted to the hospital with ARD. Paired sera were collected from all volunteers at the time of vaccination (day 0) and three weeks later (day 21). Additional blood samples were collected from volunteers admitted to the hospital with ARD.

Virus isolation was performed in primary HEK cells by standard procedures (1970 Annual Report). Type specific neutralizing antibody was measured in the same cells.

Three events prevented the study from successfully achieving its objectives. First, ARD rates did not increase as expected, so a valid test of vaccine efficacy was impossible. Second, the commercial sources of primary HEK cells could not supply sufficient usable cell cultures to test the serum pairs for an evaluation of the vaccine immunogenicity. Third, recipients of ADV-4/7/21 had higher rates of hospitalization for ARD during the first 21 days after vaccination than recipients of ADV-4/7/P. (Figure 28) The difference of hospitalization rates could not be explained by the types of viruses recovered from throat swabs since no virus isolate was obtained from most of the persons admitted to the hospital during the first 21 days. Additional field trials will be needed before the efficacy and safety of combined live adenovirus vaccination can be ascertained.

3. A search for an alternative cell culture method for adenovirus neutralization tests.

Human adenoviruses replicate best in human cell cultures. Until 1976, the DVD has relied on primary human embryonic kidney (HEK) cells from commercial suppliers as the most sensitive cells for neutralization tests in adenovirus vaccine studies. A sudden discontinuation of large scale marketing of primary HEK occurred in 1976 as the result of new federal regulations on use of human materials. As a result, the DVD developed a critical need for an alternative method of detecting type specific adenovirus antibody.

a. Alternative cell cultures. Vaccine strains of adenovirus types 4, 7 and 21 were tested in primary human amnion cells and nine continuous human cell lines. Virus titers observed were compared to those obtained earlier in primary HEK (Table 12). Of the alternative cell lines, primary human amnion and MRC-5 cells propagated all three strains of adenovirus. None of the cells, however, produced as much

virus or as distinctive a cytopathic effect as were found with primary HEK cells.

Studies are now underway to evaluate a continuous line of transformed human embryonic kidney cells (293-31) kindly supplied by Dr. Maurice Green (St. Louis University, St. Louis, MO).

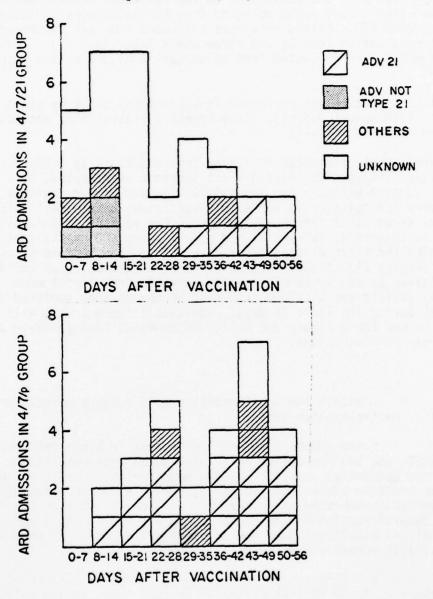


FIGURE 28. ARD admissions for ADV 4/7/21 and 4/7/P study groups by time after vaccination. Data provided by MAJ D. Johnson et al, Division of Preventive Medicine.

TABLE 12. Replication of adenovirus vaccine strains in primary and continuous human cell cultures.

Cell Culture	Dagaaga	A	denovi	rus Titer	
cerr curture	Passage Level	ADV-4		ADV-7	ADV-21
Primary cell cultures		•			
HEK		106		10 <sup>6</sup> 10 <sup>3</sup>	106 103
Human amnion		102		103	103
Continuous cell cultures					
MRC 5	29	103		104	104
NCTC 2544	266	10 <sup>3</sup> 10 <sup>3</sup>		103	No CPE*
L132			CPE	unclear	110 01 2
Girardi heart	58		CPE		
Chang conjunction	38		No	CPE	
Chang liver	•		No	CPE	
KB			No	CPE	
WISH	181		No	CPE	
Minnesota EE	.01		No	CPE	

<sup>\*</sup> cytopathic effect

#### B. Influenza surveillance

In January 1977 a routine surveillance program for influenza on BCT posts was initiated in collaboration with LTC Creed Smith, Ft. Baker, CA. The Department of Virus Diseases, WRAIR, processed specimens submitted by Forts Dix, Jackson and Knox.

Each post was requested to submit throat swabs and paired sera from 10 ARD patients per week for influenza screening. The DVD provided containers of Hanks balanced salt solution with 0.5% BSA 0.4% for collecting throat swabs to the Health and Environmental Activities Office on each post. Sera were received as pairs often weeks after the throat swab from the same individual.

Virus isolations were done by inoculation of 3-day-old embryonated eggs and harvesting the amniotic fluid 3 days later. Virus in the amnionic fluid was detected and identified by hemagglutination. Antibody was detected by a hemagglutination inhibition test using reference antigens to A/Victoria/3/75, A/NJ/8/76 and B/HK/5/72.

Between the weeks of 28 February to 25 July 77, 236 throat washes and 216 serum pairs were screened. Seventeen (17) virus isolates were recovered, and four (4) were substantiated by serological testing (Table 13).

TABLE 13. Influenza virus isolates from three BCT posts - 1977.

	Week of	Virus	HI	antibody*	
	collection	Isolate	A/NJ	A/Victoria	B/HK
Ft. Dix	18 April	A/Victoria A/Victoria	10/40 20/20	10/80 40/40	20/80 40/20
Ft. Jackson	28 March	A/Victoria " "	<10/10 40/40 <10/10	<10/20 <10/40 10/20 NOT DONE	<10/10 10/20 10/20
	18 April Unknown	B/HK A/Victoria "	<10/10	NOT DONE 20/40 NOT DONE NOT DONE NOT DONE	10/40
Ft. Knox	28 March	A/Victoria " " "	<10/<10 <10/10 <10/20 <0/40 40/20	10/20 20/40 <10/10 10/10 40/20 NOT DONE	10/20 40/40 <10/10 20/40 40/40

<sup>\*</sup> Reciprocal serum antibody titers: acute/convalescent

a.  $\underline{\mathsf{Ft.}\ \mathsf{Dix}}$ . Out of 96 throat washes received, only two virus isolates were recovered for a yield of 2%. Both were collected during the week of 18 April and were identified as A/Victoria. Specimens from Ft. Dix were routinely sent to Ft. Meade before reaching the WRAIR.

b. <u>Ft. Jackson</u>. From 76 specimens submitted, 9 isolates (12%) were recovered; 8 were A/Victoria and one was B/HK. Six isolates were obtained from throat swabs collected prior to 25 April; the other three were not dated.

c. Ft. Knox. Sixty four (64) specimens were received and 6 (9%) isolates of A/Victoria were recovered. All six strains came from throat swabs collected before 4 April.

It must be concluded that the surveillance system had several deficiencies during the first season of operation. Ft. Dix provided the most throat swabs but had the lowest virus yield; a result perhaps of the indirect route of shipping to WRAIR. Ft. Jackson provided good throat specimens but missed many pairs of sera. Nevertheless it can be concluded that influenza A strains similar to A/Victoria/75 were present on all three posts between 28 March and 25 April 1977.

### III. Miscellaneous Viruses

A. Influence of cytomegalovirus infections on the clinical outcome of renal allografts.

Serologic evidence suggests that cytomegalovirus (CMV) can be transmitted to renal transplant patients within the renal allograft (Ho, 1975). Since the presence of complement fixing (CF) antibody may indicate chronic CMV infections, some transplant centers are cautious about transplanting kidneys from donors with detectable CMV complement fixing (CF) antibodies into recipients without antibody. However, little information exists as to the actual impact CMV infection has on the two most important measures of success of renal transplantation; kidney survival and patient survival (Hanshaw, 1977).

A five year retrospective review was done of patients who received their first renal allograft between 1 May 1972, and 30 April 1977. Of 100 transplants, 80 were performed on patients who received their first transplant. Fifteen patients were excluded; three patients died before a three month followup was completed; and insufficient data was available from 12 additional patients. The total number of patients that were analyzed was 65. CMV CF data were extracted from the serologic record. In order to make the analysis as complete as possible, additional CF tests were done on serum samples of some patients. All specimens for a given patient were not run simultaneously. Serological evidence of CMV infection was defined as a sustained four-fold rise in titer in serum specimens collected before transplant and 3 to 12 months after transplant. The serological response of each patient was determined before any information regarding the clinical outcome was known.

An average of approximately two pre-transplant and five to six post-transplant CMV antibody titers were available on the 65 patients analyzed. Clinical outcomes fell into three categories; (1) dead, (2) alive, resumed dialysis, (3) alive, dialysis not required. To demonstrate the validity of an analysis using these endpoints, the outcomes of

recipients of kidneys from living related donors (LRD) were compared to recipients of cadaver donors (CAD). (See Table 14). Tables 15 and 16 summarize the data with regard to the serological response to CMV CF antigens as a correlate of clinical outcome.

TABLE 14. Effect of allograft source on outcome of renal transplantation: proportion of patients dead, on dialysis, or alive and well.

		Tim	e after t	ransplai	nt	
Clinical	1	r	2	yr	3y	r
Outcome	LRD* (46)***	CAD** (30)	LRD (39)	CAD (25)	LRD (26)	CAD (22)
Dead	.05	.17	.15	.20	.27	.20
	***	**	**	**	**	**
Alive on dialysis	.18	.50	.16	.48	.07	.53
	***	**	**	**	**	**
Alive and well	.77	.33	.69	.32	.65	.27
TOTAL	1.00	1.00	1.00	1.00	0.99	1.00

<sup>\*</sup> LRD - Living related donor of allograft

<sup>\*\*</sup> CAD - Cadaver donor of allograft

<sup>\*\*\*</sup> Number of patients followed for at least as long as the indicated "time after transplant"

<sup>\*\*\*\*</sup> P<.05 by Chi square with Yate's correction

TABLE15. Effect of recipient seroresponse\* to CMV on outcome of renal transplantation.

		Ti	me after	transpla	nt	
Clinical	13	yr	2	yr	3	yr
Outcome	CMV- (19)**	CMV+ (45)	CMV- (16)	CMV+ (39)	CMV- (13)	CMV+ (26)
					**	
Dead	.00	.07	.00	.18	.00	.31
Alive on dialysis	.37	.26	.31	.31	.31	.27
Alive and well	.63	.67	.69	.51	.69	.42
TOTAL	1.00	1.00	1.00	1.00	1.00	1.00

<sup>\*</sup> Four-fold rise in CF antibody following transplantation.

TABLE 16. Mortality of renal transplant patients as a function of CMV seroresponse.

		Total patient	
Four-fold rise in CF antibody	Number Patients	months of follow-up	Total Deaths
Absent	19	760	0
Present	46	1435	12

P < .05 by Chi square with Yates correction.

<sup>\*\*</sup> Number of patients with follow-up period equal to or longer than "time after transplant" indicated.

<sup>\*\*\*</sup> P = .05 Chi Square

These retrospective data show that a serologic response to CMV, as measured by CF antibodies, is associated with increased mortality. The association of a CMV CF seroresponse with outcome is independent of the source of the allograft; the mortality for LRD CMV- patients was 0/13; compared to 7/27 for LRD CMV+ patients.

Although patient deaths were not directly attributed to cytomegalovirus infections, CMV may be an important factor affecting the long term survival of renal transplant patients.

B. Measles - Neutralizing antibody assays and growth of vaccine virus in human blood cells.

In order to provide technical support to the Division of Medicine in a study of the effects of live measles vaccine immunization of recruits who had previously been immunized with an inactivated measles vaccine, work was initiated in two areas:

- (1) An assay for measles neutralizing antibody in a microtiter system was developed (Rosenbaum, 1963) using a substrate of Vero cells and Schwarz strain measles vaccine virus as the challenge virus. With this assay, serum neutralizing antibody titers of laboratory personnel who are known to have had clinical measles in childhood range from 1:80 to 1:640. This assay appears to be suitable for the screening of large numbers of serum specimens.
- (2) Growth of the Schwarz vaccine strain of measles virus was obtained from in-vitro cell cultures of pooled peripheral blood mononuclear cells (PBMC) recovered from heparinized blood of normal, measles immune adults (Sullivan, 1975). With 10<sup>6</sup> PBMC/ml and an MOI of 0.01, live virus could only occasionally be detected beyond two hours after innoculation in unstimulated cells, whereas PBMC which were pre-incubated for three days in phytohemagglutinin-M (PHA-M) produced virus yields of 10<sup>2</sup> to 10<sup>3</sup> TCID50/ml. A minimum concentration of PHA-M of 30ug/ml was required to obtain virus yields greater than the unstimulated control levels. Preliminary attempts to show "immune enhancement" of measles vaccine virus growth on PBMC (not split into lymphocytes and macrophage fractions) by addition of non-neutralizing concentrations of antibody were unsuccessful.
  - C. Prevalence of poliovirus neutralizing antibody in BCT trainees.

In an effort to develop data regarding the future requirements for polio immunization of basic combat trainees, a study was undertaken of the prevalence of antibodies to polioviruses types I, II and III. Single serum specimens, which were drawn from randomly selected trainees included the following: 126 sera from Ft. Jackson (22-24 March 76),

113 sera from Ft. Wood (22-24 March 76) and 47 sera from Ft. Dix (Feb 1976). Heat inactivated serum specimens were assayed by a microtiter procedure using Vero cells in medium 199 with 10% FBS. Each serum was tested at dilutions of 1:2, 1:4, and 1:8 made by use of microtiter diluting loops (Rosenbaum, 1963). Virus inocula contained 100-300 TCID50 per well of wild polio types I, II, or III. End points were read on day 3, and were defined by the degree of detectable cytopathic effect. Serum specimens with an antibody titer of less than 1:4 were interpreted as negative. Table 17 summarizes the results.

TABLE 17. Recruits lacking polio neutralizing antibody.

Basic		Trainees	without	poliovir	us Antib	ody
Training Post	Trainees Tested	Type I	Type II	Type III	A11 3	Any Type
Fort Jackson	126	9	9	11	1	22
Fort Dix	47	3	5	5	1	10
Fort Wood	113	12	9	16	1	25
Total (percent)	286(100)	24(8.4)	23(8.0)	32(11)	3(1.0)	57(20

The absence of neutralizing antibody in trainees is not necessarily equivalent to a determination of the level of susceptibility to natural infection; however, the results of this study suggest that up to 20% of trainees may be susceptible to at least one type of poliovirus, and 1.0% may be susceptible to all three.

D. Effect of Coxsackie B4 Virus infection on guinea pig embryos.

### 1. Background

The objective of this project was to determine the effect of Coxsackie B4 virus infection on the developing guinea pig embryo, particularly the cardiovascular system. There is some suggestive statistical epidemiologic evidence that infection of pregnant humans leads to congenital heart disease in the child, presumably through in-utero infection.

An animal model system was investigated to see if congenital cardiovascular malformations could be experimentally produced by embryonic infection with cardiotropic human Coxsackie B4 virus.

## 2. Study animals.

In order to obtain guinea pig embryos at various accurately timed gestational ages, guinea pigs were bred under observation. Male and female guinea pigs were caged together beginning in the late afternoon. The next morning, they were separated and vaginal smears were taken. The presence of sperm indicated that conception had occurred and the pregnancy was considered to have begun the night before.

# 3. Coxsackie B4 seed virus.

Coxsackie B4 virus was obtained from Dr. Abner Notkins (NIH). This virus was originally isolated from the myocardium of a fatal case of human myocarditis. A working virus seed was prepared in Hep-2 cell culture. Five days after infection, when CPE was marked, aliquots of culture medium and cells were harvested and frozen. This working virus seed had a titer of  $42 \times 10^7$  pfu/ml.

#### 4. Detection of virus.

A plaque assay using a human cell line, Hep-2 cells, which has been described for Coxsackie B viruses, was compared to a similar assay in LLC-MK2 cells. LLC-MK2 cells gave slightly higher titers and, because of their availability, were easier to use in the laboratory. LLC-MK2 cells were used for all subsequent assays.

#### 5. Infection of embryos.

The first step in determining the suitability of the guinea pig--Coxsackie virus model was to establish if the virus will replicate in guinea pig embryos. Adult guinea pigs are known to not be susceptible to this virus. Guinea pigs which had been pregnant for 20 days, the time of cardiac development, were anesthetized with Penthrane and a laparotomy was performed under sterile surgical conditions. The embryos were injected through the uterine wall with 1000 plaque forming units of Coxsackie B4 virus and the maternal abdomen was closed. In different animals, the embryos were collected on days 1, 3, 5 and 7 following injection of the virus. The titer of virus present in the embryos was determined by plaque assay.

## 6. Results

Virus was not recovered from any of 11 embryos tested from four different pregnant guinea pig embryos. These results are interpreted to mean that guinea pig embryos, like adult guinea pigs, are not susceptible to infection with Coxsackie B4 virus. Therefore, the guinea pig embryo will not serve as a model of congenital malformations induced by Coxsackie B4 virus.

Project 3M161102BS01 BASIC RESEARCH ON MILITARY DISEASES
Work Unit 130, Viral Infections of Man
Literature Cited.

# References:

- 1. Boyum (1968) Scand. J. Clin. Lab. Invest. 21: Supplement.
- 2. Brandt et al. (1970) J. Virol. 6:500
- 3. Brandt et al. (1967) Am. J. Trop. Med. Hyg. 16:339.
- 4. Chiewsilp and McCown (1972) Appl. Microbiol. 13:288.
- 5. Clark and Casals (1958) Am. J. Trop. Med. and Hyg. 7:561.
- 6. Eckels et al. (1976) Infect. Immun. 14:1221.
- 7. Ehrenkranz et al (1971) New Eng. J. Med 285:1460.
- 8. Griffiths et al. (1968) Amer J. Trop. Med. Hyg. 17:583.
- 9. Halstead et al. (1973) Nature New Biol. <u>243</u>:24.
- 10. Halstead et al. (1976) Proc. Soc. Exp. Biol. Med. 151:136.
- 11. Halstead and O'Rourke (1977) Nature 265:739.
- 12. Hanshaw (1977) Am. J. Dis. Child 131:841.
- 13. Ho et al. (1975) NEJM 293:1109.
- 14. Rosen (1958) Am. J. Hyg. <u>68</u>:45.
- 15. Rosenbaum et al. (1963) Proc. Soc. Exp. Biol. Med. 113:224.
- 16. Smith et al. (1970) J. Virol. <u>5</u>:524.
- 17. Sullivan et al. (1975) J. Exp. Med. 142:773.
- 18. Sung et al. (1975) Intervirol. <u>5</u>:137.
- 19. Theofilopoulous et al. (1976) J. Immunol. 117:953.
- 20. Ventura and Hewitt (1970) Amer. J. Trop. Med. and Hyg. 19:712
- 21. Winter et al. (1968) Am. J. Trop. Med. and Hyg. 17:590.

Project 3M161102BS01 BASIC RESEARCH ON MILITARY DISEASES

Work Unit 130, Viral infections of Man

Publications.

- 1. Brandt, W.E., McCown, J.M., Top, F.H.Jr., and Russell, P.K. Dengue virus replication in monocytes as a virulence marker. Abstr. Amer. Soc. Microbiol. p. 327, 1977
- 2. Catanzaro, P.J., Brandt, W.E., Hogrefe, W.R., Phillips., S.M. and Top, F.H. Jr. Virus enhanced modulation of cell surface antigens: effect of immune lytic susceptibility. J. Immunol. 117: 1104-1110, 1976.
- 3. Catanzaro, P., Agniel, L.Jr., Hogrefe, W., and Phillips, S. Interaction of peritoneal exudate lymphocytes with histocompatibility antigens. Cell. Immunol. 29: 394-402, 1977
- 4. Eckels, K.H., Brandt, W.E., Harrison, V.R., McCown, J.M., and Russell, P.K. Isolation of a temperature sensitive dengue-2 virus under conditions suitable for vaccine development. Infec. Immun. 14: 1221-1227, 1976
- 5. Theofilopoulos, A.N., Brandt, W.E., Russell, P.K., and Dixon, F.J. Replication of dengue-2 virus in cultured human lymphoblastoid cells and subpopulations of human peripheral leukocytes. J. Immunol. 117: 953-961, 1976.

	AND TECHNOLOG	Y WORK UNIT S		DA OB		7.7	10 0	1	DD-D	CONTROL SYMBOL R&E(AR)636
76 10 01	D. Change	S. SUMMARY SCTY	WORK SECURITY	NA NA	ING <sup>8</sup> Ba	NL NL	R'N	SPECIFIC	R ACCESS	9. LEVEL OF SUM A WORK UNIT
O. NO./CODES:*	PROGRAM ELEMENT	PROJECT	La	TASK AR	EA NUMBER				T NUMBE	•
- PRIMARY	62770A	3M762770		00		014	<del></del>			
b. CONTRIBUTING	61102A	3M161102	BS01			133	31			
XSOKNEHONOGX	CARDS 114F									
	stics of Atte		gue Viruse	s						
010100 Mic										
13. START DATE		14. ESTIMATED COM	PLETION DATE		G AGENCY			16. PERFOR		ТНОВ
75 07		CONT		DA				In_	House	
A DATES/EFFECTIVE:	NA	EXPIRATION:		16. RESOU	RCES ESTIMA	TE A PR	OFESSIO	MAL MAN Y	ts b Fu	105 (In thousands)
L NUMBER:*	NA	EXPIRATION:			77		3			154
G TYPE:		4 AMOUNT:		FISCAL YEAR	UNNENT	+			-	174
& KIND OF AWARD:		f. CUM. AMT.			78		3			183
9. RESPONSIBLE DOD O	PRGANIZATION	I. CUM. AMT.		20. PERFO	RMING ORGAN	ZATION		7		103
		days of D		4				T		F Passanal
walter K	eed Army Inst	itute of k	esearcn				rmy	Instit	ute o	f Research
ADDRESS: Wachi	ngton, DC 200	12			iv of C ashingt		c 20	012		
wasiii	ngton, DC 200	12		W.	asningt	on, D	L 20	012		
				PRINCIPAL	INVESTIGAT	DR (Pumiek	28 AW 11	U.S. Academi	c Inalibution	
RESPONSIBLE INDIVIDU	AL				Harris			_		
NAME: Ranmun	d, Garrison,	COI MC			me202-4	Service Transco				
	2-576-3551	COL, NO		SOCIAL S	ECURITY ACC	-	00			
I. GENERAL USE				<b>-</b>						
GENERAL USE				ASSOCIATE	INVESTIGAT	OR8				
GENERAL USE					ckels,		th H	., Dr.		
Foreign in	ntelligence n		red	HAME: E		Kenne		., Dr.		
Foreign in	BACH with Security Classifi	cation Code)		NAME: E	ckels, ummers,	Kenne Pete	r			
Foreign in	s; (U) Dengue	; (U) Vacc	ine; (U) I	MAME: E MAME: S	ckels, ummers, y; (U)	Kenne Pete Atten	r uati	on; (U	) Tiss	sue Cultu
Foreign in 12 KEYWORDS (Freedo) (U) Arbovirus 33. TECHNICAL OBJECT	EACH WIS Society Classifications; (U) Dengue	; (U) Vacc	ine; (U) In	MAME: E HAME: S	ckels, ummers, y; (U)	Kenne Pete Atten	uati	on; (U	cettan Code	ı. <b>)</b>
Foreign in (U) Arbovirus rechnical objects 23. (U) The	EACH with socially closelfings; (U) Dengue  IVE.* 24 APPROACH, 25.  objective is	; (U) Vacc PROGRESS (Pumlah II developme	ine; (U) In	mmunit;	ckels, ummers, y; (U)	Kenne Pete Atten	uati	on; (U	nuate	d vaccine
Foreign in (2) KEYBOAGS (Proceeds) (U) Arbovirus (E) YECHNICAL OBJECT (23. (U) The against class	s; (U) Dengue ve. 24 APPROACH, 28 objective is ssical strain	; (U) Vacc PROGRESS (Pumish in developments of dengu	ine; (U) Individual peragraphs id nt, product e viruses.	manue: E manue: S mmunit; milliod by mation, it	y; (U) and ass	Kenne Pete Atten ay of y pes	uati	on; (U e-atte	nuated	d vaccine of this
Foreign in (2 KEYWORDS (Proceds) (U) Arbovirus (E) YECHNICAL OBJECT (23. (U) The against classification are en	s; (U) Dengue ve. 24 APPROACH, 28 objective is ssical strain ndemic throug	; (U) Vacc PROGRESS (Pumleh II developme s of dengu hout popul	ine; (U) Individual personal led nt, produce e viruses. ated areas	mane: E mane: S mmunit; tion, The i	ckels, ummers, y; (U) and ass major to e world	Kenne Pete Atten ay of y pes , and	uati	on; (U e-atte ,3, an	nuated d 4) d mortal	d vaccines of this lity rates
Foreign in 12 KEYBORD (PRICES) (U) Arbovirus 13 YECHMICAL OBJECT 23. (U) The against classification of the virus are enare low, the	s; (U) Dengue ve.* 24 APPROACH, 24 objective is ssical strain ndemic througe e incapacitat	; (U) Vacc PROGRESS (Funded to developme s of dengu thout popul ion effect	ine; (U) In the production of	mmunit; milliod by million, The is of the	ckels, ummers, y; (U) and ass major t e world ses and	Atten Atten ay of pes and thei	uati live (1,2 alt	on; (U e-atte ,3, an hough	nuated d 4) d mortal	d vaccines of this lity rates
Foreign in 2. KEVBORDS (PROCES) (U) Arbovirus 3. YECHMICAL OBJECT 23. (U) The against clas virus are en are low, the could have a	s; (U) Dengue ve, 21 Approach, 21 objective is ssical strain ndemic throug e incapacitat serious impa	; (U) Vacc PROGRESS (Pumleh II developme s of dengu hout popul ion effect cct on mili	ine; (U) In the state of the st	mmunit: mmunition, The iof the e virus	ckels, ummers, y; (U) and ass major te world ses and and tr	Atten  Atten  ay of  y pes  , and thei	uati live (1,2 alt r as	on; (U e-atte ,3, an hough sociat ity.	nuated d 4) o mortal ed sed	d vaccines of this lity rates quelae
Foreign in (U)Arbovirus Foreimical objects Technical objects Techn	s; (U) Dengue ve, 12 Approach, 12 objective is ssical strain ndemic throug e incapacitat serious impa ected strains	; (U) Vacc PROGRESS (Purnled II developme s of dengu shout popul ion effect ict on mili are subje	ine; (U) Individual processors in the product of the viruses, atted areas and by these tary time—cted to mu.	mmunit; mmunit; mmunit; mon, a tion, a of the e virus tables ltiple	y; (U) and ass major to e world ses and and tr passag	Atten  Atten  Atten  ay of  pes  , and thei  oop mes  an	uati live (1,2 alt r as: obil d fre	on; (U e-atte ,3, an hough sociat ity.	nuated d 4) d mortal ed sed	d vaccines of this lity rates quelae
Foreign in (U)Arbovirus (U)Arbovirus (U)Arbovirus (U)Arbovirus (U)Arbovirus (U)Arbovirus (U)Arbovirus are low, the could have (24. (U) Seltissue cult	s; (U) Dengue ve. 24 APPROACH. 28 objective is ssical strain ndemic throug e incapacitat serious impa ected strains ure systems,	; (U) Vacc PROGRESS (Purnlet II developme s of dengua thout population effect tot on militiare subjet to produce	ine; (U) In mt, product e viruses, ated areas ed by these tary time- cted to mu- pure prog	mmunity tion, The pof the e virus tables ltiple eny cha	y; (U) and ass major to e world ses and and tr passag aracter	Renne Pete  Atten  Atten  Tour of open ay of  y pes  , and thei oop me es an ized	uation live (1,2 altimobil different parties)	on; (U e-atte ,3, an hough sociat ity. equent educed	nuated d 4) d mortal ed sed	d vaccine of this lity rate quelae ing in
Foreign in (U)Arbovirus FECHNICAL OBJECT  23. (U) The against classification are enare low, the could have a	s; (U) Dengue ve. 12 APPROACH, 12 Objective is scical strain ndemic througe incapacitat serious impa ected strains ure systems, tigenicity, t	; (U) Vacc PROGRESS (Purmies in developme s of dengu thout popul ion effect ict on mili are subje to produce hat will s	ine; (U) In  more recognition  e viruses,  ated areas  ed by these  tary time-  cted to mu  pure progre  erve as car	mmunity milled by make: S  The is of the e virus tables ltiple eny chandidate	y; (U) and ass world ses and tr passag aracter vacci	Kenne Pete  Atten  Atten  Text of each ay of y pes , and thei oop m es an ized i	uativities of the second of th	on; (U	muated d 4) d mortal ed sed clon: viru	d vaccine of this lity rate quelae ing in lence and
Foreign in (U)Arbovirus Foreign in (U)Arbovirus Foreign in (U) The against class virus are low, the could have seed to (U) Seltissue cultuadequate an 25. (U) 76	s; (U) Dengue ve. 24 APPROACH. 24 objective is scical strain ndemic througe e incapacitat serious impa ected strains ure systems, tigenicity, t 10-77 09 1.	; (U) Vacc PROGRESS (Purnlet II developme s of dengua hout population effect tot on militiare subje to produce hat will s A dengue-	ine; (U) In  multiple of the second of the s	mmunit; modified by mu tion, a The i of the e virus tables ltiple eny cha didate oductio	ckels, ummers, y; (U) and ass major te e world ses and tr passag aracter e vacci on seed	Atten Pete Atten ay of pes , and thei oop me es an ized ne see has	uative (1,2 altive as obil defreshed very been	on; (U e-atte ,3, an hough sociat ity. equent educed irus. prepa	nuated d 4) d mortal ed sed clon: virul	d vaccine of this lity rate quelae ing in lence and
Foreign in TREVENDE (PROCES)  (U) Arbovirus  TECHNICAL OBJECT 23. (U) The against clasvirus are elare low, the could have a could have	s; (U) Dengue IVE.* 12 APPROACH, 18. objective is ssical strain ndemic througe incapacitat serious impaected strains ure systems, tigenicity, t 10-77 09 1. optimal cond	; (U) Vacc PROGRES (Fundah Index of developme s of dengue shout population effect tet on militare subject to produce hat will s A dengue— tions for	ine; (U) Individual perspape to the notice of the viruses. ated areas ed by these tary time—cted to mulpure progerve as care 2 virus prothe mainto	mmunity wattled by nu tion, if of the e virus tables ltiple eny cha ndidate oduction	ckels, ummers, y; (U)  and ass major te world ses and and tr passag are vacci on seed of att	Atten Pete Atten ay of pes , and thei oop me es an ized ne see has enuat	uation ion ion	on; (U  on; (U	nuated 4) of mortal ed second virul red in mperat	d vaccine of this lity rate quelae ing in lence and of FRhL ture
Foreign in 12 KEYBORDA (FINISH) 1 YECHNICAL OBJECT 23. (U) The against claivirus are elare low, the could have: 24. (U) Seltissue cultissue cultis	s; (U) Dengue IVE.* 12 APPROACH, 18. objective is ssical strain ndemic througe incapacitat serious impaected strains ure systems, tigenicity, toptimal cond (i.e. low mu	; (U) Vacc PROGRES (Fundah In developme s of dengue shout popul ion effect ect on mili are subje to produce hat will s A dengue— itions for ltiplicity	ine; (U) Individual perspape to the control of the	mmunit; milled by make: S mmunit; milled by milled b	ckels, ummers, y; (U)  and ass major te world ses and and tr passag are vacci on seed of att n at 31	Atten  Pete  Atten  Atten  ay of  pes  , and thei  oop mees an  ized  ne see  has  enuat  C). U	live (1,2 altires) obil d freed v been ion	on; (U  on; (U	nuated 4) of mortal ed sec clon: virul red imperated actory	d vaccine of this lity rate quelae ing in lence and of FRhL ture y com-
Foreign in Exercise (U) Arbovirus Technical Object 23. (U) The against classification of the could have seen and the could have seen adequate am 25. (U) 76 cells under sensitivity pletion of	s; (U) Dengue ve.* 12 APPROACH, 18 objective is ssical strain ndemic throug e incapacitat serious impa ected strains ure systems, tigenicity, t 10-77 09 1. optimal cond (i.e. low mu tests for adv	; (U) Vacc PROGRESS (Pumlable developme s of dengue thout population effect ect on militare subject to produce hat will s A dengue- citions for litiplicity	ine; (U) Individual purposages is a viruses. ated areas ed by these tary time—cted to multipure progerve as care 2 virus pur the maintainput and agents and	mmunit; milled by markers. The of the e virus tables ltiple eny chandidate oduction enance growtl	ckels, ummers, y; (U)  and ass major the world ses and and tr passag aracter e vacci on seed of att at 31 uation	Atten Pete Atten A	live (1,2 althor as: obil d freed v been ion apon are	on; (U	nuated 4) of mortal ed sec clon: virul red in mperatactory all productions and productions are productions and productions and productions are productions and productions and productions are productions are productions are productions are productions and productions are	d vaccine of this lity rate quelae ing in lence and n FRhL ture y com- oduct was
Foreign in President (U) Arbovirus 1. Technical objective against classification of the could have seen are low, the could have seen adequate an 25. (U) 76 cells under sensitivity pletion of freeze-dried	s; (U) Dengue ve.* 12 APPROACH, 18 objective is ssical strain ndemic throug e incapacitat serious impa ected strains ure systems, tigenicity, t 10-77 09 1. optimal cond (i.e. low mu tests for adv d. This prod	; (U) Vacc PROGRES (Pumble to developme s of dengu- tion effect ct on mili are subje to produce hat will s A dengue- itions for litiplicity entitious uction see	ine; (U) Individual purposes, at a reas ed by these tary time—cted to multipure progerve as care 2 virus protes the maintain input and agents and d will be a served.	mmunit: milled by marker. S mmunit: milled by marker. S milled by	ckels, ummers, y; (U)  and ass major the world ses and and tr passag aracter e vacci on seed of at 31 uation or the	Atten  Atten  Atten  Ay of  yes  , and thei  oop mees an ized  ne see has  enuat  C). U  marke  prepa	live (1,2 alt of rassobil defreed very been spon spon stratic	on; (U	nuated 4) of mortal ed second virul red in mperata actory al prosubsecond subsecond red in mortal red in mperata actory al prosubsecond red in mortal red in mperata actory al prosubsecond red in mperata actory actor	d vaccine of this lity rate quelae ing in lence and n FRhL ture y com- oduct was quent lot
Foreign in Activation (U) Arbovirus Technical Objection of the Could have a could h	s; (U) Dengue ve, 21 APPROACH, 21 Objective is social strain indemic through incapacitat serious imparts acted strains ure systems, tigenicity, tagenicity, tageni	; (U) Vacce PROGRESS (Purmish in developmes s of dengue thour popul ion effect ict on mili are subje to produce hat will s A dengue- itions for ltiplicity entitious uction see ie-2 vaccin	ine; (U) Individual processors in the product of the viruses of the viruses of the virus processors of the maintainput and agents and d will be to the virus processors of the virus processors of the virus product of the virus processors of the vi	mmunity tion, The of the virus tables ltiple eny cha didate ductif enance growth atten used for	wmmers,  y; (U)  and ass major to e world ses and and tr passag aracter e vacci on seed of att n at 31 union or the enduction	Kenne Pete  Atten  Atten  Atten  Ay of  ypes  thei  oop me  es an  ized  ne see  has  enuat  C). U  marke  prepa  n lot	live (1,2 altires) obil d from the contraction of	on; (U  controlled to the limit of the limit	nuated d 4) d mortal ed sed clon: virul red in mperat actory al pro- subsec- 2 vac-	d vaccine of this lity rate quelae ing in lence and n FRhL ture y com- oduct was quent lot
Foreign in 1. KEYNOROG (PRICES) (U) Arbovirus 1. YECHNICAL OBJECT 23. (U) The against clarvirus are el are low, the could have 24. (U) Selt tissue cultisadequate and 25. (U) 76 cells under sensitivity pletion of freeze-dried of live-att been tested	s; (U) Dengue ve. 12 APPROACH. 12 objective is ssical strain ndemic througe e incapacitat serious impa ected strains ure systems, tigenicity, t 10-77 09 1. optimal cond (i.e. low mu tests for adv d. This prod enuated dengu for safety a	continuous (U) Vacce PROGRESS (Purmish in developme s of dengue thour popul ion effect ict on mili are subje to produce hat will s A dengue- itions for ltiplicity entitious uction see ie-2 vaccin nd efficac	ine; (U) In  mt, product e viruses, ated areas ed by these tary time- cted to mu pure proge erve as can 2 virus pro the maint input and agents and d will be u e. The fi y in lower	mmunitime to make it in a control of the control of	with passag and ass major te world ses and and tr passag aracter vaccion seed of att n at 31 uation or the oduction tes and	Kenne Pete  Atten ay of pes , and thei oop mes an ized in ne see has i enuat C). U marke prepa n lot a dr.	uati	on; (U	nuated 4) comortal ed second virul red in mperata actory all prosecution control of the control	d vaccine of this lity rate quelae ing in lence and n FRhL ture y com- oduct was quent loticeine has of Claime
Foreign in 1. REVISION (PRICES) (U) Arbovirus 1. YECHNICAL GRAFE) 23. (U) The against clarvirus are erare low, the could have 24. (U) Selt taken and 25. (U) 76 cells under sensitivity pletion of freeze-dried five-atte been tested Investigation	s; (U) Dengue ve.* 12 APPROACH, 12. objective is ssical strain ndemic throug e incapacitat serious impa ected strains ure systems, tigenicity, t 10-77 09 1. optimal cond (i.e. low mu tests for adv d. This prod enuated dengu for safety a onal Exemptio	(U) Vacc PROGRES (Fundah II developme s of dengue hout population effect ect on militare subject to produce hat will s A dengue- itions for itiplicity entitious uction see e-2 vaccim nd efficac n for a Ne	ine; (U) Individual perspapers to the control of th	mmunitime to make it in the street of the evirustables ltiple eny chandidation and the street of the street of the evirustable in the street of the evirustable in the street of the str	wy; (U)  and ass major te world ses and and tr passag aracter vacci on seed of att at 31 uation or the otes and s been	Kenne Pete  Atten  Atten  for operation  ypes  , and thei  oop mes and ized  has lenuat  C). U  marke  prepa  n lot  a dr.  prepa	uati live seed v (1,2 alti r as obill d fr. by reed v reed v resti	on; (U e-atte ,3, an hough sociat ity. equent educed irus. prepa and te satisf he fin. on of dengue- of "No in con	red in mperatactory all processes continued in the manual processes continued in the manual process continued in the manual pr	d vaccine of this lity rate quelae ing in lence and n FRhL ture y com- oduct was quent loti quent loti coine has of Claime nce with
Foreign in Revenue (U) Arbovirus Precunca observe 23. (U) The against clasvirus are en are low, the could have 24. (U) Self tissue cultisue cultis cultisue cultisue cultisue cultisue cultisue	s; (U) Dengue ve.* 12 APPROACH, 12 objective is ssical strain ndemic throug e incapacitat serious impa ected strains ure systems, tigenicity, t 10-77 09 1. optimal cond (i.e. low mu tests for adv d. This prod enuated dengu for safety a onal Exemptio ments for imp	(U) Vacc PROGRES (Fundah II developme s of dengue hout population effect tet on militare subje to produce hat will s A dengue titions for litiplicity entitious uction see e-2 vaccin nd efficac n for a Ne lementatio	ine; (U) Individual perspapers in the product e viruses. ated areas ed by these tary time-cted to mulpure progerve as care 2 virus protein put and agents and d will be e. The fire yin lower w Drug" (In of human	mmunitime to make it in the strict of the evirustables ltiple eny chandidatt oductive nance growth attenuated for primark ND) has trials	wy; (U)  and ass major te world ses and and tr passag aracter vacci on seed of att at 31 uation or the otes and s been s. 2. A	Kenne Pete  Atten  Atten  for open  y pes  , and thei  oop mes  es an ized  has  enuat  C). U  marke  prepa  n dr  prepa  human	uation live seed to the seed t	on; (U	red in mperatactory al prossubsector of derivation of derivation of derivation of derivative control of deriva	d vaccine of this lity rate quelae ing in lence and n FRhL ture y com- oduct was quent loti ccine has of Claime nce with ngue-3
Foreign in Precipitation of the state of the	s; (U) Dengue ve.* 12 APPROACH, 18. objective is ssical strain ndemic throug e incapacitat serious impa ected strains ure systems, tigenicity, t 10-77 09 1. optimal cond (i.e. low mu tests for adv d. This prod enuated dengu for safety a onal Exemptio ments for imp een passaged	(U) Vacc PROGRES (Fundah Me developme s of dengue thout population effect to no militiare subject to produce that will s A dengue- tions for altiplicity entitious uction see e-2 vaccin nd efficac n for a Ne lementatio and cloned	ine; (U) Individual perspapers in the produce e viruses. ated areas ed by these tary time—cted to mupure progerve as care 2 virus prothe maintainput and agents and dwill be e. The fig in lower w Drug" (II nof human in FRhL community programments and the community of the programments and the community in lower w Drug" (II nof human in FRhL community produces are virused to the community of the produces are virused to the community of the communi	mmunitiments of the evirustables ltiple eny chandidate growth attenued for primar ND) has trials ells.	y; (U)  and ass major the world ses and and tr passag and tr passag of att n at 31 uation or the odes des seen seen seen seen of att n at 30 uation or the odes odes odes odes odes odes odes ode	Kenne Pete  Atten  Atten  for open  y pes  , and thei  oop mes  an ized  ine see  has i  enuat  C). U  marke  prepa  a dr  prepa  human  human  one,	uati  live (1,2 alti r as obil d fr. by re ed v been ion ion ion ion ion ion ion ion ion io	on; (U	red in mperal actory al prossubsector of der formar of der	d vaccine of this lity rate quelae ing in lence and n FRhL ture y com- oduct was quent lot coine has of Claime nee with ngue-3 s shown to
Foreign in Parky work (U) Arbovirus (U) Arbovirus (U) Arbovirus (U) 23. (U) The against classification (U) Selection (U) Selection (U) Selection (U) Selection (U) 76.	s; (U) Dengue ve.* 12 APPROACH, 18 objective is ssical strain ndemic throug e incapacitat serious impa ected strains ure systems, tigenicity, t 10-77 09 1. optimal cond (i.e. low mu tests for adv d. This prod enuated dengu for safety a onal Exemptio ments for imp een passaged ure sensitive	(U) Vacce PROGRESS (Purellal in developmes sof dengue to militare subject to produce that will so a dengue-itions for altiplicity entitious suction see the vaccine of a new lementation and cloned in cell c	ine; (U) Individual purposes in the produce e viruses. ated areas ed by these tary time-cted to multipure progerve as care 2 virus prothe maintainput and agents and d will be ue. The firy in lower w Drug" (II no f human in FRhL coulture and	mmunity milled by makers  mmunity milled by makers  of the e virus tables ltiple eny cha addata oduction enance growth attenuased for rst pr prima nND) has trials ells. exhib	y; (U)  y; (U)	Kenne Pete  Atten  Atten  ay of  ypes  , and thei oop m es an ized ine see has enuat C). U marke prepa n lot a dra prepa huma huma one, aracte	uation in the second of the se	on; (U	red in mperal actory al prosubsec -2 vactice of der	d vaccine of this lity rate quelae ing in lence and in FRhL ture y compound to the coine has of Claimen as shown to enuation
Foreign in Parking Action 19 Present a special property of the second of	s; (U) Dengue ve.* 12 APPROACH, 12 objective is ssical strain ndemic throug e incapacitat serious impa ected strains ure systems, tigenicity, t 10-77 09 1. optimal cond (i.e. low mu tests for adv d. This prod enuated dengu for safety a omal Exemptio ments for imp een passaged ure sensitive ated into mic	; (U) Vacce PROGRESs (Pumber to development to sof denguent to produce that will so a denguention for a litiplication see the company to the	ine; (U) Individual purposes, atted areas ed by these tary time—ceted to multipure progerve as care 2 virus pretional agents and d will be the mainter of human in FRhL coulture and eys. Follow	mmunity tion, The softhie virus tables ltiple eny che didate doduction enance growth attenuated forst pro primar ND) has trials ells. exhib-	wy; (U)  and ass major to e world ses and and tr passag aracter e vacci on seed of att ation or the oductio tes and s been s. 2. A One cl ited ch accinat	Kenne Pete  Atten ay of ypes , and thei oop me es an ized ne see has lenuat C). U marke prepa n lot a dr prepa human human our aract ion w	ruati live (1,2 altir assobil d fred v bear by red v bratio of aft red of aft ired in iss labe eris	on; (U	contacted 4) of mortal ed second clone virul red in mperatactory all procesubsectice of formar of der 5, was fatter 5 clore contacted at the c	d vaccine of this lity rate quelae ing in lence and n FRhL ture y com- oduct was quent lote coine has of Claime new with ngue-3 s shown to enuation ne, mon-
Foreign in Parkey of the Action of the Actio	s; (U) Dengue ve.* 12 APPROACH, 12 objective is ssical strain ndemic throug e incapacitat serious impa ected strains ure systems, tigenicity, t 10-77 09 1. optimal cond (i.e. low mu tests for adv d. This prod enuated dengu for safety a onal Exemptio ments for imp een passaged ure sensitive ated into mic olidly protec	(U) Vacce PROGRESS (Purellal in developme sof dengue hout population effect ct on militare subject to produce hat will so A dengue-itions for altiplicity entitious suction see in e-2 vaccing and efficace of for a New lementation and cloned in cell ce and monk ted agains	ine; (U) In the product of the produ	mmunity tion, The of the virus tables ltiple eny che didate oduction enance growth atten used for ry prima ND) has trials ells. exhib wing v.	wy; (U)  and ass major to e world ses and and tr passag aracter e vacci on seed of att n at 31 uniform or the oduction tes and s been s. 2. A One cl ided ch accinat challe	Kenne Pete  Atten ay of ypes , and thei oop mees an ized ne see has lenuat C). Up marke prepa n lot a dra prepa human one, aracte ion w	ruatii live (1,2) altivate (1,2) alt	on; (U	control of the contro	d vaccines of this lity rates quelae ing in lence and n FRhL ture y com- oduct was quent lots coine has of Claimen ne with ngue-3 s shown to ne, mon- dengue-3
Foreign in Parkey of the Parke	s; (U) Dengue ve.* 12 APPROACH, 12 objective is ssical strain ndemic throug e incapacitat serious impa ected strains ure systems, tigenicity, t 10-77 09 1. optimal cond (i.e. low mu tests for adv d. This prod enuated dengu for safety a omal Exemptio ments for imp een passaged ure sensitive ated into mic	developme sof denguation effect on mili are subjet to produce hat will soft a denguations for a litition see to e-2 vaccing a deficace of the milition of the milition see to e-2 vaccing and efficace of the milition soft and efficace of the militions for a New lementation and cloned in cell conducted agains being fur	ine; (U) In our content of the maint of human in FRhL culture and eys. Follot a viremist ther purification of the maint of the maint of human in FRhL culture and eys. Follot a viremist ther purific virus products and the culture and the c	mmunity  minumity  tion,  The roof the  virus  tables  ltiple  eny cha  didate  duction  attent  used for  ryprima  ND) has  trial:  ells.  exhib  wing v.	wy; (U)  The first and asset and and tree world see and aracter aracter at 31 uation or the oduction test and see and see and see and see and see and see and tree at 21 uation or the oduction test and see and see at 2. A One clited challe a work	Kenne Pete  Atten ay of pes , and thei oop mes an ized has lenuat C). Upmarke prepa n lot a dre prepa human one, aracte ion we nged ving se	uati live (1,2 alti r as obil d obe n obe	on; (U	control of the contro	d vaccine of this lity rate quelae ing in lence and in FRhL ture oduct was quent lotterine has of Claimence with ingue-3 is shown to enaction he, mondengue-3 in prep-
Foreign in Parking of Parking and Parking	s; (U) Dengue ve.* 12 APPROACH, 12 objective is ssical strain ndemic througe e incapacitat serious impa ected strains ure systems, tigenicity, t 10-77 09 1. optimal cond (i.e. low mu tests for adv d. This prod enuated dengu for safety a onal Exemptio ments for imp een passaged ure sensitive ated into mic olidly protec C-5 clone is a live-attenu	(U) Vacc PROGRES (Fundate In developme s of dengue hout popul ion effect of militare subje to produce hat will s A dengue- itions for litiplicity entitious uction see uction see ind efficac in for a Ne lementatio and cloned in cell c e and monk ted agains being fur ated dengue	ine; (U) Individual personals in the product of viruses. ated areas ed by these tary time-cted to mulpure progerve as care 2 virus protected to mandagents and devil be en the first of human in FRHL coulture and eys. Follow the viremit ther purifice-3 vaccine	mmunitime was a minumitime with a minumitime was a minumi	wy; (U)  and assemajor the world sees and and treps aracter evaction seed of atthem at 31 uation or the oduction tes and seen so 2. A One clitted characteristic challe a work rechnice technice a work rechnice technice a work rechnice technice a work rechnice seed to the	Kenne Pete  Atten ay of pes , and thei oop mes and ized in see has in enterpreparate one, aracte in one, aracte in my end in ged in a term one, aracte in ged in ge	uati live live live live live live live liv	on; (U	control of the contro	d vaccine of this lity rate quelae ing in lence and in FRhL ture oduct was quent lotterine has of Claimence with ingue-3 is shown to enaction he, mondengue-3 in prep-
Foreign in Previous (MARDOVITUS IN TECHNICAL OBJECT) 23. (U) The against clarvirus are erare low, the could have a 24. (U) Self tissue cultissue cultiste cultiste in cultissue cultiste in cultissue cultissu	s; (U) Dengue ve.* 12 APPROACH, 18 objective is ssical strain ndemic throug e incapacitat serious impa ected strains ure systems, tigenicity, t 10-77 09 1. optimal cond (i.e. low mu tests for adv d. This prod enuated dengu for safety a onal Exemptio ments for imp een passaged ure sensitive ated into mic olidly protec C-5 clone is a live-attenu ute of Resear	(U) Vacci Process (Process of dengue to or of dengue to or of dengue to produce to or militare subject to produce to or of dengue to produce to or of tiplicity entitious for altiplicity entitious for a New lementation and cloned in cell ce and monk ted agains being fur atted dengue to Annual contracts.	ine; (U) Individual personals in the product of viruses. ated areas ed by these tary time-cted to mulpure progerve as care 2 virus protected to mandagents and devil be en the first of human in FRHL coulture and eys. Follow the viremit ther purifice-3 vaccine	mmunity  milled by metables  tion,  The control  of the  e virus  tables  ltiple  eny cha  ndidata  oductio  enance  growth  attenused for  ry prima  ND) has  trials  exhib  wing va  a when  ied as  e. Fo  eport,	y; (U)  and ass major the world see and tr passag are verified of att n at 31 uation or the oduction te do decinated the challe a work the techn 1 Jul	Kenne Pete  Atten  Atten  ay of  ypes  , and thei oop m es an ized  ne see has enuat  C). U marke prepa n lot a dra prepa huma one, aracte ion w nged ing se ical	uati  live (1,2 altir assobil d frrasi by redev by redev frati of arti of arti arti with eed repo 30 So	on; (U	control of the contro	d vaccine of this lity rate quelae ing in lence and in FRhL ture oduct was quent lotterine has of Claimence with ingue-3 is shown to enaction he, mondengue-3 in prep-

Project 3M161102BS01 BASIC RESEARCH ON MILITARY DISEASES

Work Unit 131 Characteristics of attenuated dengue viruses

Investigators.

Principal: Venton R. Harrison, M.S.

Associates: Kenneth H. Eckels, Ph.D.; Peter L. Summers

## I. Dengue-2 Vaccine Progress.

A. Passage of DEN-2, S-1 vaccine seed (FRhL-3) at 31°C. A series of passages of the S-1 seed virus was initiated at 31°C with the purpose being to select for "cold" variants, i.e., virus that would have a lower non-permissive temperature of growth after adaptation or selection at a lower temperature. In the past, passing S-1 virus at 35°C in FRhL cells was difficult because of reversion of small to large plaque virus. Reversion could be controlled to a degree by keeping the multiplicity of infection (MOI) low. However, with a low MOI, a poor yield of virus usually resulted and reversion was also seen on occasion. After several passages at 31°C, the growth and reversion frequency of S-1 virus was clearly different from past experiences with this virus at 35°C. Infectious virus yields were comparable to those at 35°C and reversion was not detectable when a relatively low MOI was used in conjunction with the low (31°C) temperature. Titers of small plaque S-1 virus after two passager at 31°C in FRhL cells are listed in Table 1. There was no evidence of reversion (large plaques at 35°C or 39.3°C when assayed in LLC-MK, cells) in any of the harvests.

Table 1. Passage of DEN-2, S-1 vaccine seed (FRhL-3 at 31°C in FRhL cells.

	Input	Vi	rus yield,	day post ino	c (PFU/0.2	m1)
Passage	virus	3	4	7	10	17
1	Und	1.4 x 10 <sup>2</sup>		3.9 x 10 <sup>4</sup>	-	-
2	Und	-	1.6 x 10 <sup>4</sup>	1.9 x 10 <sup>4</sup>	8.5 x 10 <sup>5</sup>	3.9 X 10 <sup>5</sup>
	10-2	_	$1.2 \times 10^3$	$3.5 \times 10^4$	1.9 x 10 <sup>5</sup>	7.3 X 10 <sup>5</sup>

- B. Preparation of a DEN-2, S-1 vaccine seed (FRhL-4) at 31°C. Roller flasks of FRhL cells were inoculated with DEN-2, S-1 vaccine seed (FRhL-3) and incubated at 31°C. After a media change on day 4, virus was harvested on days 6 and 10 and fluids from individual flasks were assayed for small and large plaque virus. Virus titers were low from day 4 harvests ( $\leq$  2.0 X 10 $^3$  PFU/0.2 ml), however day 10 harvests had acceptable titers (range: 7.2 X 104 to 2.9 X 105 PFU/0.2 ml). No large plaque virus was found in the day 10 harvests and only leaky, small plaque virus was detectable in plaque assays done at 39.3°C. A virus pool was made from the day 10 harvests and freeze-dried by the Department of Biologics Research. Pre-centrifuged fluids from the pool were tested for bacterial and mycoplasma as well as viral adventitious agents and were found to be free of these agents. Final container tests (after freeze-drying) including sterility and animal tests were negative. Hemadsorption tests done on the control flask 21 days after inoculation, showed no evidence of adventitious hemadsorbing agents. The titer of the seed virus (pool of 20 rehydrated vials) was 1.0 X 10 PFU/0.2 ml at 35°C with no plaques being found when assayed at 39.3°C.
- C. Growth characteristics of S-1 virus at 31°C. Table 2 lists the results of a growth curve of S-1 seed virus (FRhL-4) at 29°C, 31°C, 33°C, 35°C, 37°C, and 39°C. FRhL cell cultures were inoculated with an MOI of approximately 10<sup>-4</sup>. Temperatures of 31°C or 33°C appeared to be optimal for growth and maintenance of the small plaque population in harvests taken over a period of 7 days. This was in contrast to lower titer harvests at 35°C and 37°C where large plaque virus was found 6 and 7 days after inoculation. This is clear evidence of reversion which is dependent on temperature. One possible explanation for reversion at the low non-permissive temperature is a temperature-sensitive defect in the viral replicase which would increase the chance for mistakes to be made during transcription of viral messenger. Events of a similar nature have been found for QB phage (1), a ts mutant of respiratory syncytial virus (2), and vesicular stomatitis virus (3).

In another experiment, the effect of temperature as well as MOI on S-1 virus reversion was looked at to ascertain whether a low non-permissive temperature combined with a relatively high MOI would result in a higher incidence of revertant virus. As seen in Table 3, the only revertant virus was found in virus harvests resulting from growth at  $37^{\circ}\text{C}$  and an input MOI of 0.08. Large plaques were visible in titrations of the harvests from days 7, 8, 9, and 10, and plaqued at  $35^{\circ}\text{C}$  and  $39.3^{\circ}\text{C}$ . No revertant virus was found in flasks maintained at  $31^{\circ}\text{C}$  where titers reached over  $10^{\circ}$  PFU/0.2 ml. Virus titers were greatly reduced in flasks held at  $38.5^{\circ}\text{C}$  with the probability of detecting large plaque virus in these samples greatly minimized. Future experiments will be done using a larger number of culture flasks to increase the chance of finding revertant virus.

Effect of temperature on the replication of S-1, FRhL-4 virus in FRhL cells (MOI =  $10^{-4}$ ) Table 2.

	,		Day (PFU/m1)		
Temperature	٦	(changed media)	5	اه	/
29°C	< 5 x 10 <sup>1</sup>	5 X 10		2.2 x 10 <sup>3</sup> (s) 9.5 x 10 <sup>3</sup> (s)	1.5 x 10 <sup>4</sup> (S)
31°c	$1.5 \times 10^{2} (s)$	$6.0 \times 10^{2} (s)$	$2.3 \times 10^4 (s)  6.0 \times 10^4 (s)$	$6.0 \times 10^4 (s)$	$1.2 \times 10^{5} (s)$
33°C	$8.5 \times 10^{2} (s)$	$8.0 \times 10^{3} (s)$	$5.0 \times 10^4 (s)  9.0 \times 10^4 (s)$	$9.0 \times 10^4 (s)$	$2.2 \times 10^{5} (s)$
35°C	$7.5 \times 10^{2} (s)$	$1.3 \times 10^{3} (s)$	1.0 x 10 <sup>4</sup> (s)	$6.5 \times 10^4 (MIX)$	$5.5 \times 10^4 \text{ (MIX)}$
37°C	$2.0 \times 10^{2}$ (s)	$2.5 \times 10^{2} (s)$	$2.5 \times 10^{3} (s)$	$2.2 \times 10^4 (MIX)$	$8.0 \times 10^4 \text{ (MIX)}$
39°C	< 5 X 10 <sup>1</sup>	< 5 x 10 <sup>1</sup>	< 5 x 10 <sup>1</sup>	< 5 X 10 <sup>1</sup>	< 5 X 10

Table 3. Growth curves of S-1 vaccine seed (FRhL-4) virus in FRhL cells.

Temp.	MOI	-	2	3	4	5 6	9	7	8	6	10
31°C	0.8	<10	1.6x10 <sup>3</sup>	7.5X104	1.6x10 <sup>5</sup>	1.6x10 <sup>6</sup>	2.1X10 <sup>6</sup>	2.5x10 <sup>6</sup>	3.2x10 <sup>6</sup>	5.0X10 <sup>6</sup>	3.6x10 <sup>6</sup>
	0.08	<10	4.5X10 <sup>2</sup>	4.4x10 <sup>3</sup>	8.9X10 <sup>4</sup>	6.0x10 <sup>5</sup>	3.8X10 <sup>5</sup>	3.0X10 <sup>6</sup>	2.1X10 <sup>6</sup>	3.6x10 <sup>6</sup>	3.6X10 <sup>6</sup>
	0.008	<10	9.0X101	1.3x10 <sup>3</sup>	3.0X10 <sup>3</sup>	4.5X10 <sup>5</sup>	9.2X10 <sup>5</sup>	1.7X10	2.8X10 <sup>6</sup>	2.6X10 <sup>6</sup>	4.7X10
	0.0008		1.0X10 <sup>1</sup>	1.3x10 <sup>2</sup>	2.5X10 <sup>3</sup>	1.2X10 <sup>4</sup>	5 1.8X10	7.1X10 <sup>5</sup>	2.5x10 <sup>6</sup>	4.5X10 <sup>6</sup>	7.0X10 <sup>6</sup>
37°C	0.8	<10	3.7X10 <sup>2</sup>	$1.3 \times 10^{3}$	7.6X10 <sup>2</sup>	2.0X10 <sup>4</sup>	9.8X10 <sup>3</sup>	5.5X10 <sup>3</sup>	2.5X10 <sup>4</sup>	2.6X10 <sup>4</sup>	2.5X10 <sup>4</sup>
	0.08	<10	2.3X10 <sup>2</sup>	1.0x10 <sup>3</sup>	8.3X10 <sup>2</sup>	1.3X10 <sup>4</sup>	1.3X104 4.9X104		3.4X104*	4.1X10	4.8X10
	0.008	¢10	1.0X10 <sup>1</sup>	3.2x10 <sup>2</sup>	2.7X10 <sup>2</sup>	4.2X10 <sup>3</sup>	4.2X10 <sup>3</sup> 1.6X10 <sup>4</sup>	1.3X10 <sup>4</sup>	5.7X10 <sup>4</sup>	2.3X10 <sup>4</sup>	3.5X10 <sup>4</sup>
	0.0008	<10	<10	1.0x10 <sup>1</sup>	<10	1.2X10 <sup>2</sup>	1.2X10 <sup>2</sup> 2.3X10 <sup>2</sup>	5.1X10 <sup>2</sup>		1.5X10 <sup>4</sup>	5.2X10 <sup>4</sup>
38.5°C	9.0	<10	4.0X101	2.0X10 <sup>1</sup>	2.0X10 <sup>1</sup>	3.1X10 <sup>2</sup>	2.0X10 <sup>2</sup>	2.6X10 <sup>3</sup>	3.3X10 <sup>3</sup>	2.3X10 <sup>2</sup>	3.2X10 <sup>3</sup>
	0.08	<10	1.0x101	5.0x10 <sup>1</sup>	¢10	2.5x10 <sup>2</sup>	5.3x10 <sup>2</sup>	2.7x10 <sup>2</sup>	4.6X10 <sup>3</sup>	1.9x10 <sup>3</sup>	1.8x10 <sup>3</sup>
	0.008	<10	<10	<10	<10	7.0X10 <sup>1</sup>	4.0X101	¢10	7.1X10 <sup>2</sup>	3.5X10 <sup>3</sup>	5.6X10 <sup>2</sup>
	0.0008	<10	<10	<10	<10	<10	1.0X10 <sup>1</sup>	<10	2.0X10 <sup>1</sup>	1.5X10 <sup>3</sup>	3.0X10 <sup>1</sup>
39.3°C	8.0	<10	<10	1.0x101	<10	3.0x10 <sup>1</sup>	1.0X101	5.0X101	7.0x10 <sup>1</sup>	5.0x10 <sup>1</sup>	<10
	0.08	<10	<10	,<10 10	<10	<10	2.0X10 <sup>1</sup>	2.0X10 <sup>1</sup>	1.6X10 <sup>2</sup>	8.0X10 <sup>1</sup>	6.0X10 <sup>1</sup>
	0.008	<10	<10	<10	<10	¢10	2.0X101	¢10	3.0X10 <sup>1</sup>	3.0X10 <sup>1</sup>	¢10
	0.0008	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10

<sup>\*</sup> Harvests containing large plaque, revertant virus.

On day 10 post inoculation, the flasks that had been held at 39.3°C were shifted down to 31°C to see whether infectious virus could still be released from these cells. As listed in Table 4, released virus reaching a titer of approximately 10<sup>4</sup> PFU/0.2 ml was detected 2 days post shift down to 31°C. Only the flask that received an input MOI of 0.0008 failed to respond to the shift down. From this experiment it appears that virus, in some stage of replication, can persist in cultures incubated at 39.3°C. The temperature effect is reversible and does not result in permanent impairment of a functional protein.

Table 4. S-1 virus yields from infected cells held at 39.3°C for 10 days, then shifted down to 31°C for 3 days.

	Day, PFU/C	.2 ml (after shif	t to 31°C)
MOI	1	2	3
0.8	7.9 X 10 <sup>3</sup>	8.3 x 10 <sup>3</sup>	1.5 X 10 <sup>4</sup>
0.08	6.3 X 10 <sup>3</sup>	1.4 x 10 <sup>4</sup>	1.9 x 10 <sup>4</sup>
0.008	$8.8 \times 10^{2}$	$4.1 \times 10^3$	4.1 X 10 <sup>3</sup>
8000.	< 10	< 10	< 10

D. Response of monkeys to DEN-2, S-1 vaccine, Lot 1. To evaluate the efficacy of the DEN-2 vaccine, Lot 1, 5 Rhesus monkeys were inoculated with the vaccine, each receiving 0.5 ml subcutaneously. Following inoculation, each monkey was bled on 10 consecutive days, followed by bleedings on days 15, 30, and 45. From Table 5, virus was recovered from only one monkey on one day following inoculation with the vaccine. All 5 monkeys stimulated CF, HI, and neutralizing antibodies following inoculation with neut titers ranging from 10 to 220 for the monkeys. After a period of 7 months, all 5 monkeys were bled again then challenged with DEN-2, GM-6 parent virus or DEN-2, Asian strain #21868. The Asian strain of DEN-2 was included as a challenge virus to see if the S-1 vaccine, which is a Puerto Rican isolate would stimulate protection against a heterologous strain of DEN-2. Of the 2 monkeys that received the Asian virus, one had a viremia that lasted 3 days; one out of 3 monkeys that received the homologous challenge virus also had a viremia lasting 3 days. Monkeys that had low or undetectable levels of antibody responded to the challenge with rapidly escalating antibody titers. Monkey 853 which had the highest pre-challenge antibody titers, had only a 3-fold rise in neut antibody. Previously challenged monkeys inoculated with the S-1 subline passed in PGMK cells were 100 percent protected against homologus parent virus and showed little or no rise in antibody

Table 5. Immune response of monkeys to DEN-2, S-1, vaccine Lot #1

Monkey		Post	vacc 1/C	4	1001	TOSE VACE I/III		יחפר ווכתר
No.	Viremia	15 day	15 day 30 day	30 day 45 day	15 day	30 day	45 day	45 day
867	No	16	16	32	20	20	20	20
810	No	œ	∞	16	<10	10	10	10
853	1 day	32	32	32	20	20	40	200
792	No	16	32	32	20	20	40	220
179	No	16	16	16	<10	10	10	100

\* inoc = 3.8 X 10<sup>4</sup> PFU

Table 6. Response of DEN-2 vaccinated monkeys after challenge.

Monkey						15 da	15 days post	30 day	30 days post			
No.	Challenge virus	Viremia	- A	re chal	1		chal1	U	chal1	45 day	vs post	chall
			1/CF 1/HI Neut 1	1/HI	Neut	1/CF	1/HI	1/CF	1/HI	1/CF	1/CF 1/HI Neut	Neut
198	DEN-2, Asian #21868	No	7>	<10	<10 <10 1024	1024	320	512	079-	512	-640	70000
810	(0 A 10" FFU)	3 days	7>	10	<10	256	-640	256	-640	128	-640	2400
853		No	80	07	250	79	64 160	1/9	80	16	80	700
792	DEN-2, PR-159, parent	No	7>	<10	07	256	-640	79	160	79	80	2330
179	( OT V C)	3 days	4>	10	09	32	-640	256	-640	128	320	4100

following challenge (4). In these same studies, antibody titers were significantly higher following primary inoculation. In the section to follow, the question of immunogenic alteration of the S-1 virus after passage in FRhL cells will be discussed.

E. Comparison of S-1 virus after PGMK passage and FRhL passage. Experience with the DEN-2, S-1 vaccine in monkeys prompted an evaluation of the temperature sensitive characteristics of the vaccine virus in cell culture and in suckling mice. The vaccine which is the result of 4 passages of the S-1 subline in FRhL cells was compared to S-1, pl9a virus which was passed exclusively in PGMK cell cultures. Table 7 lists the efficiency of plating (EOP) values of the Lot 1 vaccine and S-1, p-19a viruses using PFU titers from assays done at  $35^{\circ}$ C and  $38.5^{\circ}$ C. Also listed are the suckling mouse IC  $LD_{50}/PFU$  ratios which indicate the degree of attenuation of these viruses. A higher EOP or SMIC  $LD_{50}/PFU$ value would indicate less temperature sensitivity for the virus and most likely less attenuation in vivo. There is less than 2 two-fold difference in these ratios for the FRhL-passed virus as compared to the PGMK (S-1, p-19a) passed virus and the higher value is found for the FRhL-passed virus. This does not correlate with the results of monkey inoculation with the vaccine where it appears to be more attenuated. Further evaluation of the temperature sensitivity of the vaccine virus will be done in the future to account for the monkey inoculation experiments.

Table 7. In vitro and in vivo characteristics of DEN-2 virus passed in PGMK cells and in FRhL cells.

	PFU/O.	2 ml	EOP	
Virus	35°C	38.5°C	38.5°C/35°C	SMIC LD <sub>50</sub> /PFU
S-1, FRhL-4 (Vaccine Lot 1)	1.5 x 10 <sup>5</sup>	3.2 X 10 <sup>3</sup>	0.021	0.002
S-1, p-19a	$1.4 \times 10^{6}$	1.6 x 10 <sup>4</sup>	0.011	0.0012

## II. Dengue-3 Vaccine Progress.

A. Passage and cloning of DEN-3, CH53489 virus in FRhL cells. Previous passage of the DEN-3 human serum isolate CH53489 in PGMK cells resulted in a parent virus pool at the fourth passage level in these cells. In order to adapt the virus to FRhL cells, a substrate (considered acceptable) for human vaccine preparation, passages of the PGMK parent virus were made in these cells at regular intervals of 10 to 12 days. The results of 6 passages of this virus are listed in Table 8. Temperature sensitivity decreased with passage in the FRhL cells, starting with an approximate 20-fold difference in plaque titers at 35°C and 39.5°C(pass 1) to an approximate 5-fold difference after 6 passes. This was the reverse of what occurred previously upon passage of a similar virus in FRhL cells (Annual Report, 1975), in which the same human isolate, passed 4 times in PGMK, then 6 times in FRhL cells showed a definite increase in temperature sensitivity upon passage.

Table 8. Passage of DEN-3, CH53489, PGMK-4 virus in FRhL cells.

morphology	Plaque me	2 m1	PFU/O	Day of	
t 35°C	at :	39.5°C	35°C	harvest	Passage
e of large all plaques		3.2 x 10 <sup>2</sup>	7.2 X 10 <sup>3</sup>	11	1
11	"	2.3 X 10 <sup>2</sup>	3.2 X 10 <sup>3</sup>	12	2
п	"	8.0 X 10 <sup>1</sup>	2.0 X 10 <sup>3</sup>	10	3
"	"	$3.8 \times 10^2$	$1.6 \times 10^{3}$	10	4
и	"	4.0 X 10 <sup>3</sup>	2.3 X 10 <sup>4</sup>	10	5
"	"	6.8 x 10 <sup>3</sup>	$3.6 \times 10^4$	10	6

The FRhL passage 6 virus (also labelled parent) was used in cloning experiments to attempt selection of naturally-occurring temperature sensitive or attenuated variants. The FRhL-6 virus contained mixed populations of large, medium, and small plaques which appeared to be separable by cloning. After several unsuccessful attempts to plaque the FRhL-6 virus under agarose overlays, an alternate method proved to be feasible, that consisted of terminally diluting the FRhL-6 virus so that a small number of wells of a 24 well cell culture plate could be infected. With inputs of 11 PFU/well

and 6 PFU/well, 7 and 2 wells were respectively infected in 2 cell culture plates with 24 well capacities. The results of this cloning experiment are found in Table 9. Most of the harvests from individual wells contained mixtures of large and small or medium and small plaques. However, clones labelled C-5 and D-2 appeared to contain pure small plaque virus and clone B-5 contained mostly large plaque virus. The clones containing large plaque virus had temperature sensitivities resembling the parent virus while the C-5 clone had a reduction in plaque titer of > 2.0 logs at 39.5°C.

Table 9. Clones of DEN-3 derived by terminal dilution of parent, FRhL-6.

			<del>,</del>	
ar especial	Clone	35°C PFU	39.5°C	Plaque morphology
	A-3	2.9 X 10 <sup>4</sup>	8.0 x 10 <sup>3</sup>	Large & small
	B-3	2.7 X 10 <sup>4</sup>	$1.5 \times 10^{3}$	Medium & small
	B-5	1.8 x 10 <sup>4</sup>	6.0 x 10 <sup>3</sup>	Large & small
Plate 1	C-4	3.1 X 10 <sup>4</sup>	4.2 X 10 <sup>3</sup>	Large & small
(11 PFU/well)	C-5	$7.0 \times 10^3$	< 5 X 10 <sup>1</sup>	Small, faint
	D-1	2.9 X 10 <sup>4</sup>	2.0 X 10 <sup>2</sup>	Medium & small
	D-2	2.0 X 10 <sup>3</sup>	$3.0 \times 10^2$	Small, distinct
Plate 2	C-2a	3.2 X 10 <sup>4</sup>	1.6 x 10 <sup>4</sup>	Large & small
(6 PFU/well)	C-4a	3.7 X 10 <sup>4</sup>	9.5 x 10 <sup>3</sup>	Large & small
	parent (FRhL/6)	2.2 X 10 <sup>4</sup>	6.5 x 10 <sup>3</sup>	Large & small

Seed pools of each clone were made after passing one more time in FRhL cells and were used for cloning at the passage 9 level. Prior to cloning at the passage 9 level, all inocula were passed through a 0.2 M Nalgene filter to remove viral aggregates and lessen the probability of inoculating more than one virus particle per well. Steps in the cloning procedure are found in Table 10 for 4 of the original 9 clones first isolated at the passage 7 level. These were selected as representative of the plaque size variants found after the first cloning passage. For

Table 10. Procedure for isolating DEN-3 clones.

		Passa	Passage: procedure	ie.		Ь	PFU/m1	EOP (PFU)
Clone	7:cloning	8:seed prep	9:cloning	10:cloning	Clone 7:cloning 8:seed prep 9:cloning 10:cloning 11:seed prep 35°C 39.3°C	35°C	39.3°C	39.3°C/35°C
B-3	(F & S)*	(F & S)* (L & S)	(8)	(8)	(s)	1.0 x 10 <sup>5</sup>	1.0 x 10 <sup>5</sup> <1.5 x 10 <sup>2</sup> >0.0015	>0.0015
B-5	(F & S)	(F & S)	(F & S)	(T)	(L)	1.9 X 10 <sup>4</sup>	1.9 X 10 <sup>4</sup> 4.3 X 10 <sup>3</sup> 0.23	0.23
C-5	(s)	(S)				5.5 x 10 <sup>3</sup> <5.0	<5.0	6000.0
D-1	(M & S)	(M & S)	(M & S)	(H)	æ	1.3 x 10 <sup>5</sup>	1.3 x 10 <sup>5</sup> <1.5 x 10 <sup>2</sup> >0.0012	>0.0012
FRhL-11,	/WD: Human s no clon	FRhL-11/WD: Human serum isolate passed 11 times in FRhL cells; no cloning; plaque size: S	bassed 11 tin (ze: S	nes in FRhL ce	11s;	9.5 x 10 <sup>4</sup>	9.5 X 10 <sup>4</sup> <1.5 X 10 <sup>2</sup> >0.0016	>0.0016

\* L = large size plaques (approx. 2.0 mm)
M = medium size plaques (approx. 1.0-2.0 mm)
S = small size plaques (approx. <1.0 mm)

B-3, D-1, and B-5, terminal dilution plating techniques were successfully employed in isolating homogeneous populations of either small, medium, or large plaque viruses. The C-5 clone, passage 11, was low in titer and after several attempts to optimize virus yields, this line for C-5 was abandoned and another started after passage of the pass 8 virus two more times in DRhL cells. After these two passages, the C-5 clone appeared to be better adapted to growth in FRhL cells and another cycle of 3 clonings was initiated and will be reported on in the future.

An additional line of passages was initiated with the DEN-3 human serum isolate which resulted in 11 passages in FRhL cells exclusively. This study was done by D.R. Dubois, Department of Biologics Research, and reported on in detail in another Annual Report. The virus at the eleventh passage level, labelled FRhL-11/WD, contained small plaque virus and appeared to be temperature sensitive in plaque assays (Table 10). The FRhL-11/WD virus was further characterized without purification along with the four DEN-3 clones.

B. Growth characteristics of DEN-3 clones in FRhL cells. Growth curves were initiated with clones B-3, B-5, C-5, D-1, FRhL-11/WD, and the parent (FRhL-6) viruses at temperatures of 33°C, 37°C, 38.5°C, 39.3°C, and 40.0°C. The plaque titers for samples taken daily for 10 days are listed in Table 11. All clones maintained their original plaque morphology in culture samples taken over the 10 day period. The DEN-3 parent virus grew at all temperatures tested, with the greatest reduction of virus titers occurring in flasks incubated at 39.3°C and 40.0°C. Growth of the B-5 clone most closely resembled the parent virus, although no growth of B-5 was observed at 40.0°C. The C-5 clone replicated only at 33°C and 37°C while the other viruses were intermediate in their ability to replicate at the higher temperatures. Table 12 summarizes the differences in plaque titers between the permissive temperature up 33°C and the higher temperatures of growth for day 8 of the growth curve.

Table 12. Differences in virus yields for DEN-3 clones grown at permissive (33°C) and higher temperatures (37°C, 38.5°C, 39.3°C, 40°C)

		Log10 d	ifferen	ce in t	iter, da	y 8	
Virus titer at	FRhL-6	B-3	B-5	C-5	D-1	y 8 FRhL-11/WD	
33°C-37°C	0.32	1.12	0.41	1.39	1.00	-0.41	
-38.5°C	1.07	2.68	1.63	>3.54	2.48	1.83	
-39.3°C	1.69	2.28	2.70	>3.54	3.53	>3.11	
-40.0°C	2.55	>4.60	>3.81	>3.54	>4.38	>3.11	

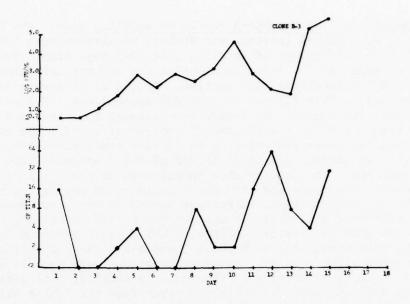
Table 11. Growth curves of DEN-3 clones in FRhL cells.

					1	Day, PFU/0.2 ml	0.2 ml				
Virus	Temp.	1	2	3	3 4	5	9	7	80	6	10
FRhL-6	33°C	1.0x10 <sup>2</sup>	3.4X10 <sup>2</sup>	1.3X10 <sup>3</sup>	8.8X10 <sup>3</sup>	1.0X10 <sup>2</sup> 3.4X10 <sup>2</sup> 1.3X10 <sup>3</sup> 8.8X10 <sup>3</sup> 2.1X10 <sup>4</sup> 1.0X10 <sup>5</sup> 7.2X10 <sup>4</sup> 1.4X10 <sup>5</sup>	1.0x10 <sup>5</sup>	7.2X10 <sup>4</sup>	1.4X10 <sup>5</sup>	3.9X104	3.7X10 <sup>4</sup>
(parent)	37	2.0X10 <sup>1</sup>	2.2X10 <sup>2</sup>	3.5X10 <sup>2</sup>	1.2X10 <sup>3</sup>	2.2X10 <sup>2</sup> 3.5X10 <sup>2</sup> 1.2X10 <sup>3</sup> 1.8X10 <sup>4</sup> 2.1X10 <sup>4</sup>	2.1X104	8.2X10 <sup>3</sup>	8.2X10 <sup>3</sup> 6.8X10 <sup>4</sup>	9.4X10 <sup>3</sup>	6.2X10 <sup>3</sup>
	38.5	<10		1.0x10 <sup>2</sup>	2.5x10 <sup>2</sup>	3.0X10 <sup>1</sup> 1.0X10 <sup>2</sup> 2.5X10 <sup>2</sup> 1.6X10 <sup>3</sup> 5.1X10 <sup>3</sup>	5.1X10 <sup>3</sup>	4.3X10 <sup>3</sup> 1.2X10 <sup>4</sup>		2.0X10 <sup>3</sup>	1.0x10 <sup>3</sup>
	39.3	<10	1.0X101	1.0X10 <sup>1</sup>	1.5x10 <sup>2</sup>	$1.0 \times 10^{1}$ $1.5 \times 10^{2}$ $1.4 \times 10^{3}$ $1.6 \times 10^{3}$ $1.0 \times 10^{3}$	1.6X10 <sup>3</sup>	1.0x10 <sup>3</sup>	2.9X10 <sup>3</sup>	2.2X10 <sup>3</sup>	4.5x10 <sup>2</sup>
	07	<10	<10	<10		2.0x101 2.0x101 9.0x101	9.0X10 <sup>1</sup>	2.0X10 <sup>1</sup>	2.0x10 <sup>1</sup> 4.0x10 <sup>2</sup> 2.2x10 <sup>2</sup>	2.2X10 <sup>2</sup>	4.0X10 <sup>1</sup>
Clone B-3 33°C	33°C	<10	1.0x101	1.3X10 <sup>2</sup>	1.2X10 <sup>3</sup>	1.3X10 <sup>2</sup> 1.2X10 <sup>3</sup> 9.4X10 <sup>3</sup> 1.5X10 <sup>4</sup>	1.5x10 <sup>4</sup>	2.0X10 <sup>5</sup> 4.0X10 <sup>5</sup>		1.3X10 <sup>5</sup>	1.2X10 <sup>5</sup>
	37	<10	<10	2.0X101	1.2x10 <sup>2</sup>	$2.0 \times 10^{1} \ 1.2 \times 10^{2} \ 3.5 \times 10^{3} \ 8.3 \times 10^{3} \ 6.6 \times 10^{3} \ 3.0 \times 10^{4}$	8.3x10 <sup>3</sup>	6.6X10 <sup>3</sup>	3.0X104	1.5X10 <sup>4</sup>	5.7X10 <sup>3</sup>
	38.5	<10	2.0X10 <sup>1</sup>	<10	<10	2.0X10 <sup>1</sup>	1.1X10 <sup>2</sup>	1.1x10 <sup>2</sup> 1.0x10 <sup>2</sup>	8.4X10 <sup>2</sup>	7.4X10 <sup>2</sup>	4.3X10 <sup>2</sup>
	39.3	<10	<10	<10	<10	6.0X10 <sup>1</sup>	1.2x10 <sup>2</sup>	2.8x10 <sup>2</sup>	2.1X10 <sup>3</sup>	2.4X10 <sup>3</sup>	3.6x10 <sup>2</sup>
	07	<10	<10	<10	<10	<10	<10	<10	¢10	<10	<10
Clone B-5 33°C	33°C	<10	<10	7.01101		6.0X10 <sup>2</sup> 4.4X10 <sup>3</sup>	3.0x10 <sup>4</sup>	5.0X10 <sup>4</sup>	6.5X104	4.8X10 <sup>4</sup>	4.8X10 <sup>4</sup>
	37	<10	<10	1.0x101		2.9X10 <sup>3</sup>	2.9X10 <sup>3</sup> 6.7X10 <sup>3</sup> 7.6X10 <sup>3</sup>	7.6X10 <sup>3</sup>	2.5x10 <sup>4</sup>	4.5x10 <sup>3</sup>	1.5x10 <sup>4</sup>
	38.5	<10	<10	<10	2.0X10 <sup>1</sup>	4.0X101	8.0X101	1.8X10 <sup>2</sup>	$8.0 \times 10^{1} 1.8 \times 10^{2} 1.5 \times 10^{3} 1.3 \times 10^{2}$		5.0x101
	39.3	<10	<10	<10	<10	<10	4.0X10 <sup>1</sup>	$4.0 \times 10^{1} 1.5 \times 10^{2} 1.3 \times 10^{2}$		2.6x10 <sup>2</sup>	1.0x10 <sup>2</sup>
	07	<10	<10	<10	<10	<10	<10	×10	<10	<10	<10

Table 11. Growth curves of DEN-3 clones in FRhL cells (continued)

					ď	Day, PFU/0.2 ml	2 m1				
Virus	Temp.	1	2	3	4	5	9	7	8	6	10
Clone C-5	33°C	410	1.0x101	410	<10 3.0x10 <sup>2</sup>	1.1X10 <sup>3</sup>	1.2X10 <sup>4</sup>	9.4x10 <sup>3</sup>	3.5X10 <sup>4</sup>	3.1X104	1.2X10 <sup>4</sup>
	37	410	410	<b>410</b>	<b>410</b>	1.2x10 <sup>2</sup>	3.0X10 <sup>2</sup>	3.5x10 <sup>2</sup>	1.4X10 <sup>3</sup>	8.0X10 <sup>2</sup>	2.0X10 <sup>2</sup>
	38.5	<b>^10</b>	<10	410	°10	410	<10	<10	<10	410	410
	39.3	<10	<10	<10	410	<b>410</b>	<10	<b>410</b>	¢10	<10	<10
	07	<10	<10	<10	<10	410	<10	<10	<10	<10	<b>د10</b>
Clone D-1	33°C	410	1.3x10 <sup>2</sup>		5.5X10 <sup>2</sup> 1.8X10 <sup>3</sup>	1.1X10 <sup>4</sup>	7.4X10 <sup>4</sup>	1.1110	2.4X10 <sup>5</sup>	1.6X10 <sup>5</sup>	7.1X104
	37	<10	1.0X101		2.0X101 9.0X101	2.5X10 <sup>3</sup>	6.6x10 <sup>3</sup>	5.4X10 <sup>3</sup>	2.4X104	4.7X10 <sup>3</sup>	4.3X10 <sup>3</sup>
	38.5	<10	<10	<10	1.0X10 <sup>1</sup>	1.9x10 <sup>2</sup>	2.5x10 <sup>2</sup>	2.9x10 <sup>2</sup>	8.0X10 <sup>2</sup>	3.9X10 <sup>2</sup>	3.4 X10 <sup>2</sup>
	39.3	<10	<10	<10	<10	<10	<10	2.0X10 <sup>1</sup>	7.0X10 <sup>1</sup>	1.6X10 <sup>2</sup>	2.0X10 <sup>1</sup>
	07	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
FRhL-11/WD	33°C	¢10	8.0X10 <sup>1</sup>	1.0X10 <sup>1</sup>	1.1X10 <sup>2</sup>	1.2x10 <sup>3</sup>	1.4x10 <sup>3</sup>	2.2x10 <sup>3</sup>	1.3X104	6.8x10 <sup>3</sup>	5.4X10 <sup>3</sup>
	37	<10	7.0x10 <sup>1</sup>	<10	<10	3.2x10 <sup>2</sup>	2.2X10 <sup>3</sup>	3.1X10 <sup>3</sup>	3.3X104	8.8X10 <sup>3</sup>	1.5x10 <sup>3</sup>
	38.5	<10	<10	<10	<10	1.0x10 <sup>1</sup>	<10	1.0X101	1.9x10 <sup>2</sup>	1.7X10 <sup>2</sup>	6.0x10 <sup>1</sup>
	39.3	<10	<10	<10	<10	<10	<10	<10	<10	<10	¢10
	40	<10	<10	<10	<10	<10	<10	<10	¢10	<b>10</b>	<10

- C. Replication of the DEN-3 clones in suckling mice. The DEN-3 clones along with FRhL-6 (parent) and FRhL-11/WD viruses were inoculated intracerebrally into groups of suckling mice (2-3 days old). Two mice were removed each day following inoculation for recovery of infectious virus and complement-fixing (CF) antigen. Brains of mice were removed and homogenized in EMEM diluent to give 20% suspensions. Following clarification, the supernatant fluids were assayed for virus and CF antigen. Figure 1 shows the PFU and CF antigen titers for the mouse brain homogenates taken from mice up to 18 days post-inoculation. Surprisingly, CF antigen was found in all of the inoculated mice one day after inoculation. The antigen then appeared to have a cyclic appearance which in some cases coincided with the appearance of infectious virus. In the case of the C-5 clone, infectious virus, at very low levels, was found only on 2 days while CF antigen was easily recovered, especially after 11 days post-inoculation. Signs of CNS disease and some deaths occurred in mice inoculated with the B-3 and B-5 clones as well as the FRhL-6 and FRhL-11/WD viruses. These observations were made 14 days or more after inoculation of virus. Virulence in suckling mice serves as a marker to differentiate the C-5 clone virus from the FRhL-6 parent virus. The nature of the early CF antigen will also be studied for comparison to the dengue soluble CF antigens.
- D. Selected DEN-3 viruses in rhesus monkeys. Three DEN-3 virus seeds that were relatively temperature resistant and had mixed plaque populations were inoculated, along with the C-5 clone (homogeneous small plaque, temperature sensitive virus), into rhesus monkeys. Serum samples were assayed for virus on a daily basis for 10 days; serum taken at intervals of 15, 30, and 45 days was tested for CF and HI antibodies. Nine of ten monkeys that received the temperature resistant virus developed viremias lasting at least 2 days (Table 13). Both monkeys that were inoculated with the C-5 clone were free of viremia but still responded with significant levels of CF and HI antibodies. After holding the C-5 inoculated monkeys for 8 months, they were challenged with the DEN-3, FRhL-6 virus. No viremia was detected for 10 days following challenge (Table 14). Serum CF and HI antibody levels did not change over the 8 month holding period while antibody titers in both monkeys increased following challenge. Three other DEN-3 clones and the FRhL-11/WD virus were also tested in rhesus monkeys for virulence. These viruses, except for B-5, showed some degree of temperature sensitivity in cell cultures. In monkeys, however, all of these viruses produced viremias lasting 1 to 4 days (Table 15). Clone B-3 appeared to be the most attenuated in this limited study with 1 of 2 monkeys developing a viremia that lasted 2 days. All clones and the FRhL-11/WD virus stimulated approximately the same levels of CF and HI antibodies 45 days following inoculation.



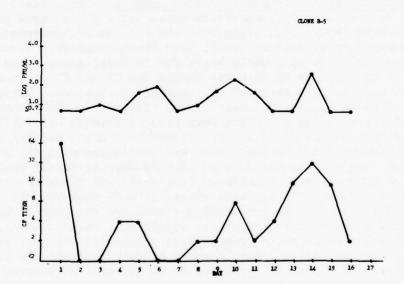
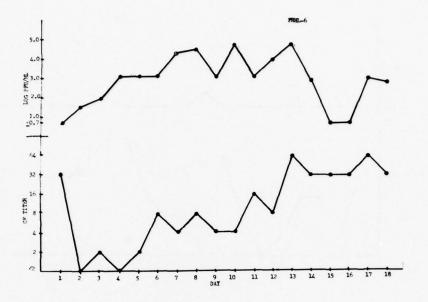


Figure 1. Infectious virus and CF antigen recovered from suckling mouse brains after inoculation with DEN-3 clones.



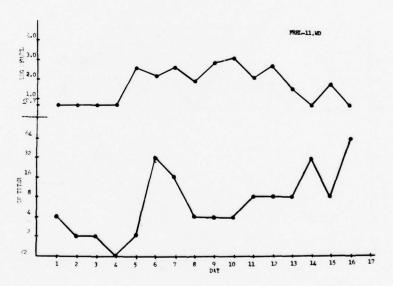
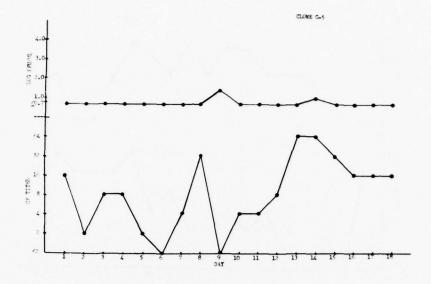


Figure 1. (continued)



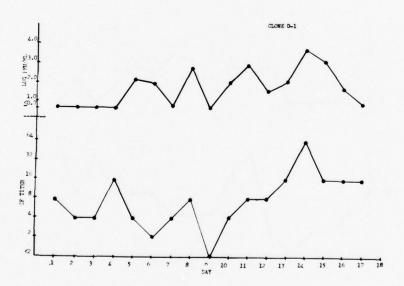


Figure 1. (continued)

Table 14. Challenge of rhesus monkeys 8 months after primary inoculation with DEN-2, C-5 clone.

45 days 1/HI	160	320
Post-chall:4 1/CF	79	16
:30 days 1/HI	326	320
Post-chall:30 days Post 1/CF 1/HI 1/C	79	32
Post-chall:15 days 1/CF 1/HI	320	320
Post-chall	128	128
ha11 1/HI	07	160
Pre-cha 1/CF	32	79
No. days viremia	0	0
Monkey Challenge No. days no. inoc. viremia	1.7 x 10 <sup>5*</sup>	1.7 x 10 <sup>5*</sup>
Monkey no.	331	332

\* Challenge virus: DEN-3, FRhL-6 (parent); 0.5 ml subcutaneously.

Table 13. Response of rhesus monkeys to selected DEN-3 viruses.

Viria nassage	Inoculum			VIr	Viremia	(PFL	(PFU/m1)					CF HI	HI
Topics Lead 1		-	7	6	4	2	9	-	00	6	10	day 30	day 30
D-3, PGMK-4	7 x 10 <sup>4</sup>	1	•	2	01	1			,	t	- 1	79	160
		- 1	1	1	ſ	1	30	~	1	•	1	79	160
	9 x 10 <sup>3</sup>	1	1	1	10	30	2	•	1	ı	1	128	160
		1	1	1	•	10	30	1	1	,	,	128	160
D-3, FRhL-6	4 X 10	1	10	1	1	10	20	1	1		1	128	80
		1	1	ı	ı	1	10	2	5		1	79	160
D-3, FRhL-8	3 x 10 <sup>4</sup>			- 1	ı		•	ı	1	,	1	128	320
		1	1	ı	S	30	20	10	1	,	•	128	160
	4 x 10 <sup>3</sup>	1	1	1	i	•	1	1	1	10	15	128	160
		1	15	1	1	20	1	1	1	1	1	128	160
D-3, clone C-5	3 x 10 <sup>3</sup>			1	1		1		1	ı	1	32	07
		ı	,	1	1	1	•	ι	ı	1	,	99	160

Table 15. Inoculation of rhesus monkeys with selected clones of DEN-3 virus.

		Geome	etric mean
No. monkeys viremic/total	Average no. days viremia	antibody 1/CF	1/HI
2/2	3.5	91	320
1/2	1.0	64	320
2/2	3.0	64	227
2/2	4.0	64	320
	2/2 1/2 2/2	viremic/total     days viremia       2/2     3.5       1/2     1.0       2/2     3.0	No. monkeys viremic/total         Average no. days viremia         antibody 1/CF           2/2         3.5         91           1/2         1.0         64           2/2         3.0         64

The rhesus monkey appears to be very sensitive to infection with DEN-3 virus. Only the C-5 clone which is highly temperature sensitive (cut-off temperature of 38.5°C) did not produce a viremia. However, with antibody levels remaining constant over a period of 8 months, a limited infection with the C-5 clone must have been established which may have persisted for the observed period.

Project 3M1611102B\$01 BASIC RESEARCH ON MILITARY DISEASES
Work Unit 131 Characteristics of attenuated dengue viruses
Literature Cited.

## References:

- 1. Valentine, R.C., Ward, R., and Strand, M. The replication cycle of RNA bacteriophages. p. 1-59. IN: K.M. Smith, M.A. Lauffer, and F.B. Bang (ed.), Advances in Virus Research, Vol. 15, 1969. Academic Press, Inc., New York.
- 2. Schnitzer, T.J., Richardson, L.S., and Chanock, R.M. Growth and genetic stability of the ts-1 mutant of respiratory syncytial virus at restrictive temperatures. J. Virol. 17: 431-438, 1976.
- 3. Flamand, A. Genetical behavior of vesicular stomatitis virus during successive passages at high and low temperatures. Mutation Research  $\underline{17}$ : 177-184, 1973.

#### Publications:

1. Eckels, K.H., Brandt, W.E., Harrison, V.R. McCown, J.M., and Russell, P.K. Isolation of a temperature-sensitive dengue-2 virus under conditions suitable for vaccine development. Infect. Immun. 14: 1221-1227, 1976.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY			DA OA 6443			2. DATE OF SUMMARY 77 10 01		DD-DR&E(AR)636	
B. DATE PREV SUM'RY 4. KIND OF SUMMARY   S. SUMMARY SCTY* 6. WORK SECURITY			DA C			SO'N INSTR			D. LEVEL OF SUM
76 10 01	D. Change	u u	NA.			NI.	CONTRACTO YES	NO NO	A WORK UNIT
10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	_		NUMBER	1	WORK UNI		R
& PRIMARY	61102A	3M161102BS01	-	00			132		
b. CONTRIBUTING			1						
C. CONTRIBUTING	CARDS 114F		<del>                                     </del>			1			
	Security Classification Code							******	
		of Military Importance	e						
	CHNOLOGICAL AREAS								
010100 Micr	obiology								
13. START DATE		14. ESTIMATED COMPLETION DATE	IS FUN	DING A	GENCY		16. PERFORM	ANCE ME	гнор
58 05		CONT	DA			1	C. In	-Hous	e
17. CONTRACT/GRANT			14. RES		S ESTIMAT	E & PROF	ERSIONAL MAN YE	s b Fu	NDS (In thousands)
A DATES/EFFECTIVE:	NA	EXPIRATION:		1				1	20
F NUMBER:*			FISCAL	77			6	4	28
C TYPE:		d AMOUNT:	YEAR	1			9	1	-0
& KIND OF AWARD:		f. CUM. AMT.		78			9	0	58
Un 1 to			1		G ORGANI				
NAME: Walter	keed Army In	stitute of Research	HAME:*				ny Instit	ute o	f Researc
	DO 000	212			of C		2 20212		
ADDRESS: Washin	igton, DC 200	012	ADDRES	s:•Wa	shing	con, Do	20012		
							AN II U.S. Academi		9
Perposible INDIVIDU		COI MC	NAME: Formal, Samuel B. Ph.D.						
TELEPHONE: 202	l, Garrison, ( 2-576-3551	COL, MC	TELEPHONE: 202~576-3758 SOCIAL SECURITY ACCOUNT NUMBER:						
TELEPHONE: 202	. 570-5551		4				R:		
GENERAL USE					Tram		. Sadoff,	A C	ross
Foreign in	telligence no	ot considered	NAME:		T L CHILL	, 0	· badorr,	0	2000
22. KEYWORDS (Precede	BACH with Security Classifi	Cotton Code) (U) Pseudomonas	S aer	1101	nosa.	(II) Ne	eisseria	menin	offidis.
(II) Local im	munity to had	cteria; (U) Immunolog	v: (11	I) A	ntibi	tics:	(U) Infe	ction	s Disease
		PROGRESS (Furnish Individual personaphe Ide iology, ecology, epide							
		ic aspects of diseases							
		litary forces. Curren						ningo	coccal,
		and staphylococcal in							
		cteriologic techniques							
		actic regimens, spread	d and	pe	rsist	ence of	organis	ns in	various
military pop					1.				- 6
		tive cell wall complex							
		mammalian cells. A ne							
		inhibit elongation fac							
		tween core structure							
		her enterobacteriaceae			0		and the second second		
		ated. A factor elabor							
		tivity of PMN's was fo							
		toxin levels to pseudo							
		with antibody rises to							
		opsonic. Quantitativaccess site infection							
		access site infection rmy Institute of Resea							
30 Sep 77.	alter Reed A	imy Histitute of Research	arch	Ailli	ual I	Ogresa	, Keport,	1 00	1 70
50 бер //.									
		495							

Project 3M161102BS01 BASIC RESEARCH ON MILITARY DISEASES

Work Unit 132 Bacterial Diseases of Military Importance

Investigators.

Principal: Samuel B. Formal, Ph.D.

LTC Jerald Sadoff, MD, MC; LTC Edmund C. Tramont,

MD, MC

Associates: MAJ Alan Cross, MD, MC; William C. Branche, Jr.,

Ph.D.; MAJ George Lowell, MD, MC; Arthur S. Dobek, Ph.D.; Gerald Pier, Ph.D.; Jennie Ciak; Edward T. Dickson, Jr.; SP5 Wavell Hodge; Lynette Smith;

Kathryn Yamada; Hazel Sidberry

- 1. Purification procedures were developed for isolation of high molecular weight lipopolysaccharide-free polysaccharides from Pseudomonas aeruginosa. These were found to contain an alkali-labile determinant against which active and passive immunization was directed.
- 2. Ninety percent of clinical isolates of <u>Pseudomonas aeruginosa</u> (PA) were found to produce toxin A. Solid phase binding antibody and neutralizing antibody against toxin A was found following colonization or non-fatal infection with PA but was absent in fatal septicemia. The toxin is capable of inhibiting macrophage and lymphocyte function.
- 3. A new pseudomonas toxin (toxin S) was discovered. It inhibits protein synthesis by ADP-ribosylation of elongation factor 1.
- 4. The immunochemical relationship between core structures in the LPS of the J-5 mutant of  $\underline{E}$ .  $\underline{coli}$  0111 and LPS from other enterobacteriaceae  $\underline{P}$ .  $\underline{aeruginosa}$  and  $\underline{N}$ .  $\underline{meningitidis}$  was determined.
- 5. Bacteremic patients respond to the LPS of the infecting organism, often with antibody levels exceeding 50  $\mu g/ml$  .
- 6. Lymphocytes, upon stimulation by PHA, produce a factor released into the supernatant fluid that is able to favorably influence bacterial kill by polymorphonuclear leukocytes.
- 7. While all three immunoglobulin classes were capable of inducing antibody-dependent mononuclear cell-mediated antibacterial activity against meningococci, IgA (although it did not fix C') was most effective with cells while IgM (although far more efficient than IgG in cooperation with C') was least efficient.
- 8. Monocytes and Null (k) lymphocytes (but not T or B lymphocytes) were effective in antibody-dependent cell-mediated system in cooperation with post-immunization sera or purified convalescent IgG.

- 9. Among normal sera, antibody-dependent cell-mediated activity is more prevalent than is complement-mediated killing of meningococci.
- 10. Quantitative blood cultures simplify diagnosis and management of access site infection in patients undergoing chronic hemodialysis.
- 11. A modification of the test for inhibition of bacterial attachment to epithelial cells was unsuccessful.
- 12. Preliminary results suggest that pili and native cell wall complex are both involved in epithelial cell attachment.
- 13. A variety of tests were performed on specimens from patients from WRAMC. These included the isolation of <u>Treponema pallidum</u>, 92 clinical specimens for special isolation, counter-current immunoelectrophoresis for specific bacterial antigens, radioimmunoassay determination for teichoic acid antibody and antigen, and the Limulus assay for endotoxin.
- Techniques were developed for isolating high molecular weight polysaccharides from the slime layers of Pseudomonas aeruginosa Fisher-Devlin immunotypes I and II. The techniques were directed toward purification of these polysaccharides with minimal contamination from toxic lipopolysaccharides. Following alcohol precipitation procedures and cetavalon precipitation to remove nucleic acid contamination, the polysaccharides were subjected to acetic acid hydrolysis for 14 hours to cleave contaminating lipopolysaccharide into its lipid A and polysaccharide components. Chloroform extraction removed the remaining lipid; cold phenol extraction removed remaining protein; and the high molecular weight polysaccharides were subsequently collected in exclusion volume of a Sephadex G-100 column. Removal of further trace nucleic acid contamination was accomplished by ion exchange chromatography. The polysaccharides from immunotypes I and II did not stick to anion or cation exchange resins over a wide ph range while nucleic acids have a high affinity for DEAE resins.

The chemical composition of purified Type 1 polysaccharide as compared to lipopolysaccharide (LPS) extracted by hot phenol water from type I organisms is shown in table 1. As would be expected the lipopolysaccharide contained considerably more lipid. The polysaccharides were free of protein and had only minimal nucleic acid contamination prior to ion exchange chromatography. The immunochemical relations between these purified polysaccharides and lipopolysaccharides isolated from homologous organisms by standard Westphal techniques were examined by Ouchterlony and solid phase radioimmunoassay techniques. Stearyl chloride derivatization of the polysaccharides did not change their behavior in Ouchterlony gel diffusion experiments and greatly facilitated their sticking to the soft microtiter plates used in the solid phase procedures.

Rabbit antisera raised either by hyperimmunization with live organisms or crude slime preparations gave two lines in Ouchterlony

gels when reacted with either crude slime or lipopolysaccharides: one slow migrating line and one more rapidly migrating line. Reaction of these sera with purified polysaccharide yields a single line. This single line gave a reaction of identity with the rapidly moving line seen with cruder preparations. These results have been interpreted to mean that lipopolysaccharide preparations are contaminated with polysaccharide thus yielding two lines while the purified polysaccharide contain very little lipopolysaccharide. Fisher-Devlin lipopolysaccharide vaccines were likewise tested and shown to be contaminated with polysaccharides. Attempts to purify lipopolysaccharides free from contaminating polysaccharide by deoxycholate chromatography have been only partially successful. Acetic acid hydrolysis of Westphal purified lipopolysaccharide with subsequent Sephadex G-100 chromatography yielded three polysaccharide fractions, two in the high molecular weight range and one below 50,000 daltons. The high molecular weight fractions gave lines of identity with the purified polysaccharide. These results suggest either that a large amount of high molecular weight polysaccharide contaminates lipopolysaccharide preparations, or that high molecular weight polysaccharides may represent high molecular weight-0 side chains of LPS. Previous experiments with deoxycholate G-100 chromatography and SDS polyacryamide gel electrophoresis, however, had suggested that the lipopolysaccharides were in the 12,000 dalton range.

Although the antigenic determinant on the polysaccharide was stable in mild acid it was found to be alkali labile. Treatment with 0.1 N HaOH at 25° for 2 hours destroyed the determinant when assayed for by the Ouchterlony technique. This property was utilized in solid phase radioimmune inhibition assays to demonstrate that sera could be prepared which contained antibody excluvisely against the polysaccharide determinant. The solid phase reaction between polysaccharide and rabbit antisera raised against crude preparations or live organisms could be inhibited by either polysaccharide or lipopolysaccharide. This was expected because as was discussed previously all lipopolysaccharide preparations had been shown to be contaminated with high molecular weight polysaccharides. The reaction between polysaccharide and rabbit antisera could not however be inhibited by alkali treated LPS. This suggested the means for obtaining a rabbit sera from which anti LPS antibody had been removed. When rabbit antisera was absorbed with alkali treated LPS it no longer reacted with LPS in the solid phase but continued to react in high titre with purified polysaccharide.

Passive protection experiments in mice demonstrated that 0.2 ml rabbit anti-polysaccharide type I sera absorbed with alkali treated LPS given intraperitoneally 1 hour prior to challenge with 10-50  $\rm LD_{50}$  was able to protect 90% of the animals. Active vaccination with purified type I polysaccharide preparations followed 7 days later by intraperitoneal challenge with 10-100  $\rm LD_{50}{}^{\circ}{}_{\rm S}$  yielded between 40-80% protection. Results of some of these experiments are shown in table 2. Alkali treated polysaccharide did not elicit active protective responses in mice while alkali treated LPS still retained this property. The inability of alkali treated polysaccharides to induce protection further

suggests that the protection seen with polysaccharide vaccine was not due to LPS contamination, because LPS retains its immunogenicity following alkali treatment. This series of experiments suggests that an alkali labile determinant against which active and passive immunization is directed can be found in purified high molecular weight, LPS free, polysaccharides from Pseudomonas aeruginosa immunotype 1.

2. The role of pseudomonas toxin A in human disease was studied in collaboration with Dr. Barbara Iglewski from the University of Oregon. Several large batches of toxin A were purified. A new purification technique utilizing immunoabsorbent chromatography was developed. Antitoxin A was coupled to Sepharose 4B by cyanogen bromide techniques. It was found that single band toxin A could be eluted with 2M potassium iodide after crude preparations had been allowed to equilibrate with the column and extensively washed with 0.01 M Tris pH 7.5.

Solid phase radioimmunoassay procedures for quantitation of class specific antibody against the toxin were developed. In addition a Chinese Hamster Ovary (CHO) cell neutralization assay technique was also developed to measure functional neutralizing antitoxin. These procedures were then used to measure antibody in 85 sera from 56 patients infected with Pseudomonas aeruginosa, and 55 sera from normal soldiers and hospital matched controls. The results indicated that normal subjects have neutralizing and measurable IgG antibody against the toxin, but these levels rarely exceed 1:8-1:16 neutralizing titre, or 1-2  $\mu$ gm/ml of IgG, and less than 1  $\mu$ gm/ml IgM or IgA antibody. Three of 25 normal soldiers had less than 5 µgm/ml of specific IgG antitoxin. Colonized patients make considerable neutralizing antibody (1:8-1:1204) and up to 20-40 µgm specific IgG. Four patients who survived serious pseudomonas infections produced high levels of antibody and three patients who died of E. coli sepsis also had high levels of neutralizing and IgG antibody. Strikingly 10 of 11 patients who died of pseudomonas sepsis had very low or no neutralizing or IgG antibody (1:8 or less neutralizing antibody and less than 1 µgm IgG). These low levels could indicate general inability to respond or perhaps to consumption of antibody.

In a parallel study, the incidence of toxin A production by other pseudomonas species was studied. Ninety percent (100/111) of clinical and laboratory <u>Pseudomonas aeruginosa</u> species investigated were toxin A producers. None of 28 non aeruginosa pseudomonads were producers. These included strains of <u>P. fluorescens</u>, <u>P. multophilia</u>, <u>P. pseudomallei</u>, <u>P. sutida</u>, and <u>P. cepacia</u>.

It was demonstrated that toxin inhibited protein synthesis as measured by uptake of radiolabelled amino acids in isolated human transfer macrophages, T, B and Null cells. The macrophages were found to be most sensitive to the toxin (80-90% inhibition 3 days after exposure to this toxin). Blast transformation by lymphocytes as

measured by thymidine uptake was inhibited by the toxin. The ability to inhibit efferent and afferent limbs of the immune system further suggests a role for toxin A as a virulence factor in pseudomonas disease.

- The molecular mechanism of action of toxin A is known to be similar to that of diptheria toxin, involving the ADP ribosylation of Elongation Factor 2 (EF-2). Crude filtrates from P. aeruginosa strain 388 originally isolated from a burn unit in Seattle neither reacted with antitoxin A antisera in gel precipitation, nor could its toxicity for CHO cells be neutralized by anti A. These culture filtrates, however, were active in the ADP ribosylation assay, i.e. radiolabelled nicotimanide adenine dinucleotide (NAD) in the presence of strain 388 filtrate and wheat germ or rabbit reticulocyte extract was converted from trichloracetic acid (TCA) soluble to TCA insoluble label. When this reaction was studied in greater detail it was found that the strain 388 filtrate (toxins) was a true NAD-ase and that ADP-Ribose was being transferred. This was determined by following the fate of label in different portions of the NAD molecule during the reaction. Only label in the adenine and ribose portions was transferred while nicotinamide was not. When purified EF-2 was used as a receptor rather than wheat germ no transfer of label occurred. When 90% pure EF-1 was used as a receptor, quantitative transfer of label was observed. It was therefore considered that toxin A and toxin S had different substrates. Under limiting conditions of wheat-germ-EF2 and in the presence of excess toxin A (further addition of toxin A could not increase transfer), addition of toxin S doubled the transfer further further proving distinct substrates for the two toxins. When the reaction products of toxin A and toxin S were run in SDS-PAGE, the gels frozen, sliced, eluted, TCA precipitated and counted, the characteristic 100,000 Dalton EF-2 product was seen to contain toxin A. The product of the toxin S reaction gave radioactive peaks at 50,000 and below which were somewhat characteristic of EF-1 (figure 1). The mechanism of action of toxin S may be EF-1+NAD toxin S ADP-R-EF1+H+.
- 4. Studies on the immunochemical relationship between the J-5 mutant of  $\underline{E}$ .  $\underline{\operatorname{coli}}$  Olll and other gram negative lipopolysaccharides (LPS) have continued. Solid phase inhibition experiments had indicated that there were shared determinants between the J-5 mutant and LPS from a variety of enterobacteriaceae,  $\underline{P}$ .  $\underline{\operatorname{aeruginosa}}$ , and  $\underline{N}$ .  $\underline{\operatorname{meningitidig}}$ . Direct experiments were performed. Pre and post-immunization sera from rabbits vaccinated with J-5 were tested directly for their ability to bind to LPS from heterologous organisms. Tables 3 and 4 show the consistent rises in antibody against LPS from a variety of organisms including 10 LPS types of meningococcus and 6 of 7 immunotypes of  $\underline{P}$ .  $\underline{\operatorname{aeruginosa}}$ . These results suggest that J-5 may represent an all purpose anti-endotoxin vaccine. It has already been safely given to more than a hundred volunteers in studies by Dr. A. Braude, University of California, San Diego, CA.
- 5. Since it has been shown that members of the enterobacteriaceae and Pseudomonas aeruginosa (PA) share a common core structure in the

lipopolysaccharide (LPS) of their outer membrane, it has been suggested that antibody to these core determinants may prevent shock and death subsequent to bacteremia in both human patients and animals. Therefore the human antibody response to bacteremia with these organisms was measured by a functional (opsonic) and binding (SPRIA) antibody assay to the homologous LPS of the infecting organism to the LPS of the rough J-5 mutant of E. coli 0111 and to the LPS of the Re mutant of Salmonella minnesota. Antibody levels of 10 µg ml or more were detected in convalescent sera from 5 of 18 patients against J-5 LPS, 3 of 16 patients against the salmonella Re LPS and 12 of 16 patients against the homologous LPS. (8 of these 12 had levels of greater than 50 µg m1.) Antibodies of IgG, IgA and IgM classes were all produced. The actual levels varied greatly from patient to patient and did not seem to correlate with patient survival, and the time of rise of antibody also varied greatly. Most patients had significant elevations in titer by the end of the first week. In the 3 patients who have been studied, opsonic antibody appeared when there were high levels of binding antibody. This opsonic antibody was inhibited by the homologous LPS at 10 μg/ml in one patient bacteremic with E. coli and at 50 μg/ml in a second patient bacteremic with Salmonella enteritidis. Both Re and J-5 LPS in final concentration of 50  $\mu g/mI$  failed to inhibit opsonic antibody in any patient tested. Pre and post-vaccination sera to J-5 from healthy human volunteers and from hyper-immunized rabbits were also compared for differences in opsonic capability. No differences were found.

- 6. As a corollary to the question of the role of opsonic antibody response to prognosis in gram negative bacteremia, stimulation of lymphocytes was studied to determine whether this would lead to enhanced bacterial killing by PMN's. Supernatants from PHA stimulated lymphocytes influence PMN's to kill more efficiently as determined by bacterial kill and chemilumenescence. This enhanced bacterial killing by PMN's occurs in the absence of complement and antibody, since neither the supernatant nor PHA was bactericidal.
- 7. It has been demonstrated that both lymphocytes and monocytes (either together or when separated) can, in cooperation with group specific human immune sera, effect antibody-dependent cell-mediated antibacterial activity (ADCAA) against group C meningococci (Mgc). We also have shown that this ADCAA could be abrogated by adsorbing groupspecific antibodies from the antisera using homologous group C Mgc polysaccharide (Psss). No effect was noted when using homologous protein or LPS antigens or heterologous polysaccharide. We now report studies on the effect of purified human IgG, IgM and IgA in our system. Purified IgG, IgM and IgA were prepared as previously described from patients convalescing from group C type meningococcal disease. A representative experiment showing the ability of IgG to be effective in ADCC is shown in figure 2. Since we showed that all immunoglobulin activity in the sera used was directed against Psss, we could quantitate the amount of Ig effective in our system using a radioactive binding assay. As shown

in table 5, significant ADCC activity was present when as little as 4 antigen-binding units (ABU) of IgG were used. IgG was also effective with complement (C') down to this level. IgM, in contrast, was effective with C' when as little as 0.2 ABU of IgM were used while it was effective with cells only using quantities > 3.2 ABU, a 16-fold difference (table 6). Since IgA was shown to block IgG-mediated C'dependent lysis of Mgc, experiments were performed to determine if IgA could block IgG-mediated cell-dependent killing; it did not (table 7). Moreover, as shown in table 8, although IgA could not kill Mgc at all in cooperation with C', it was effective in ADCC even when as little as 0.6 ABU of IgA were used. In summary, as shown in figure 3 and table 9, IgA could mediate ADCC of Mgc using 10 fold less ABU of immunoglobulin than either IgG or IgM even though it could not kill in cooperation with C'. Furthermore, IgA-mediated ADCC was comparable in magnitude to that mediated by IgG. Both IgG and IgA were more effective than IgM in ADCC although in cooperation with C', IgM was at least 16fold more effective than IgG. This observation suggests a role for IgA in anti-bacterial cell-mediated immunity, particularly, in areas where complement levels are low or ineffective.

- We have suggested that lymphocyte (and/or monocyte) mediated, complement-independent antibacterial system may be analogous to the recently described mechanism of antibody-dependent cellular cytotoxicity (ADCC) which has been shown to be mediated by lymphocytes and/or monocytes. Accordingly, we examined the nature of the effector cells which mediate ADCAA. Mononuclear cell populations were prepared as shown in figure 4. The ability of these cell populations to mediate ADCC of Mgc in cooperation with post-Csss immunization is shown in table 10. Indeed, polymorphonuclear leukocytes, monocytes & Null (k) lymphocytes were found to be effector cells in our system. Similarly, these cells, but not purified B or T lymphocytes, were effective in cooperation with IgG purified from  $C_{ ext{II}}$  convalescent sera (table 11). These results tend to emphasize the similarity between our cell-mediated anti-bacterial activity and previously described lymphocyte and monocyte mediated ADC effector mechanisms and suggest a role for the null (k) cell in cell-mediated antibacterial immunity.
- 9. We examined the role of ADCAA in natural immunity by comparing cell-mediated to complement-mediated antibacterial activity in pre-and post-Csss immunization sera. As shown in table 12 and figure 5, normal human peripheral blood mononuclear cells were capable of killing group C Mgc in cooperation with heat-inactivated normal adult sera in the absence of added complement. Moreover, this bactericidal activity mediated by non-adherent mononuclear cells was significantly more prevalent among normal sera than was complement-mediated killing. Furthermore, following immunization with Mgc group Csss, significant rises in titer and magnitude of both cell-medited and C'-mediated killing were induced resulting in C' + Ab activity equal to or greater than ADCC-type killing. It was also determined that both lymphocytes and monocytes contribute to the ADCC-type bactericidal activity seen with both normal and Csss immune sera. These observations suggest

a role for antibody-dependent cell mediated antibacterial activity in natural immunity, particularly in those individuals lacking antibody-dependent complement-mediated activity.

- 10. Quantitative counts on both blood from peripheral veins and access sites (AS) of patients undergoing chronic hemodialysis have been performed in an effort to simplify diagnosis of AS infections. Blood from both sites was cultured by pour plate technique in (1) patients suspected of having AS infection on clinical grounds and (2) from patients with normal-appearing AS. All 35 patients were followed for the duration of hospital stay (and for most, during subsequent hospitalizations). Quantitative cultures were able to differentiate between patients with AS infections and those whose AS was sterilein spite of clinical signs of infections. Moreover, we were able to predict when conservative management of AS infection with antibiotics would fail.
- 11. Previous studies have demonstrated the usefulness of the test for inhibition of epithelial cell attachment (IEA) by bacteria as a measurement of "functional antibody", especially for local antibodies. However, this test as described in these studies required much time and was extremely tedious. Also, the procedure required the use of benzidine in one step; this is a potential carcinogen, which should be avoided if possible. Therefore an attempt was made to modify and simplify the test. The IEA test was performed as previously described, except for the following modifications (a) the organisms were radiolabelled with I<sup>135</sup>; (b) separation of epithelial cells with attached organisms from free (non-attached) organism was attempted by using gradients (ficoll, sucrose); and (c) the number of organisms was quantitated using radiolabelled specific antisera. Despite a great deal of effort adjusting these parameters, the modifications were not found to be helpful. Radiolabelling of organisms resulted in loss of antigen (s) mediating attachment (presumably pili). Radiolabelling of antisera did not adequately differentiate attached organisms on epithelial cells probably because of non-specific attachment of the radiolabelled globulin to the epithelial cells. However, during the course of these studies it was determined that quantitation of the number of organisms attached per cell was a sensitive method of determining antibody function.
- 12. Since it was determined that antibody could block bacterial attachment to epithelial cells, attention was turned to identifying those antigen (s) mediating this effect. Two antigens which might be involved in attachment were tested; pili and native cell wall complex. Neisseria pili were supplied by Dr. Charles Brinton, University of Pittsburgh. Neisseria native complex was made as previously described. The IEA procedure was employed in a competitive test using progressively increasing concentrations of antigen. In addition, vaginal secretions were absorbed with pili to determine if the inhibiting effect of antibody in secretions would be removed. Both pili and native complex inhibited homologous and heterogenous strains in a dose response fashion. Homologous inhibition was usually more intense (table 13). Neither E. coli pili nor pseudomonas native complex had any effect. Adsorption

of a human vaginal secretion with the homologous pilus preparation blocked the inhibition. Thus, both pili and native complex antigens were capable of blocking IEA. This property appears to be species specific. Since native complex probably contains the pilus antigen it is not yet known if cell wall components other than pili are also involved in attachment.

The Department of Bacterial Diseases continues to perform specialized microbiologic tests for military patients. There were 3 unsuccessful attempts to isolate  $\underline{T}$ . pallidum from syphilitic patients using procedures previously described. Twenty meningococcal isolates were received for serological confirmation. Of those surviving transportation 5 were group B, 6 were group Y, and one each of group 135 and 29E. No group C isolates were received. Ninety-two specimens from humans, chimpanzees and dogs were received for special isolate attempts; 31 throat cultures, 3 stool, 12 urine and 4 tissue specimens. Fifteen gentamicin blood level determinations were done. Two hundred and twenty-five specimens were tested for the presence of staphylococcal teichoic acid antibodies using counter current immunoelectrophoresis (CIE). The specimens included six cerebrospinal fluids, two joint fluids, and 117 sera. Except for the meningitidis cases, the specimens were from WRAMC patients suspected of having staphylococcal infections. Sera from 17 hospital staff, 10 from patients with non-staphylococcal infections, 14 from Ft. Ord recruits and five from gram-negative patients sepsis were tested as negative controls. Forty of the 225 specimens were found to have teichoic acid antibodies. Two hundred and ten of these specimens were also analyzed by radioimmunoassay for IgG, IgA and IgM levels to teichoic acid antigen prepared in this laboratory. There was significant titer increase for IgG in most cases, but usually no increase in IgM and IgA titers. CSF from patients with bacterial, viral or fungal meningitidis have been reported to have characteristic amines, acids, and alcohols apparently contributed by the metabolism of the invading microorganism. The extreme sensitivity of gas-liquid chromatography allows the detection of minute quantities of the derivatized biochemicals. Thus far CSF obtained from non-infected WRAMC patients have been analyzed to obtain a normal baseline for the amine derivatives. The CSF from 2 cases of meningitis show distinct differences from the normal. Commercially available limulus amoebocyte lysate may be used to detect the presence of as little as 1 nanogram or less of endotoxin. This test has recently been instituted in this laboratory and is presently being standardized.

Project 3M161102BS01 BASIC RESEARCH ON MILITARY DISEASES
Work Unit 132 Bacterial diseases of military importance

# Literature Cited.

### References:

- 1. Tramont, E. C., Griffiss, J. M., Rose, D. L., Brooks, G. F., Artenstein, M. S. Strain Differentiation of <u>Neisseria</u> gonorrhoeae. J. Infect. Dis. 134:128-134, 1976.
- 2. Tramont, E. C. Specificity of Inhibitions of Epithelial Cell Adhesion of Neisseria gonorrhoeae. Infect. & Immun. 14: 593-595, 1976.
- 3. Artenstein, M. S., Brandt, B. L., Tramont, E. C., Zollinger, W. D. Immune Response <u>Neisseria meningitidis</u>. Noel Rose, Herman Friedman Editors, pps. 274-279, in Manual of Clinical Immunology, 1976.
- 4. Tramont, E. C. Current Concepts in the Management of Bacterial Meningitidis. Mil. Med. 141:589-594, 1976.
- 5. Tramont, E. C. Persistence of <u>Treponema pallidum</u> in Cerebrospinal Fluid Following Recommended Penicillin G. Therapy. JAMA 236:2206-2207, 1976.
- 6. Cross, A. S., Tramont, E. C. Fever of Unknown Origin. Mil. Med. 141:761-763, 1976.
- 7. Tramont, E. C. Inhibition of Adherence of Neisseria gonorrhoeae by Genital Secretions. J. Clin Invest., 59:117-124, 1977.
- 8. Tramont, E. C., Sadoff, J. C., Wilson, C. Variability of the Lytic Susceptibility of Neisseria gonorrhoeae to Human Serum. J. Immunol., 118:1843-1851, 1977.
- 9. Tramont, E. C., Wilson, C. Variation in Buccal Cell Adhesion of Neisseria gonorrhoeae. Inf. & Imm., 16:709-711, 1977.
- 10. Stark, F. R., Branche, W. C., Jr., Collins, T. and Engelkirk, P. Staphyloccal Infections. New Methods of Defining Epidemic Conditions and Results of Therapy. The Brunn Method of Therapy. Sixteenth Interscience Conference Sixteenth Interscience Conference Session 13, October, 1976.
- 11. Raines, David R., Branch, W. C. Jr., Anderson, W. C., Daniel, L. and Boyce, Worth, H. The Occurrence of Bacteremia after Esophageal Dilation. Gastroenterology 17:1183, April, 1976.

- 12. Lowell, G. H., Smith, L. F., and MacDermott, R. P. Lymphocyte and Monocyte-Mediated Antibody-Dependent Killing of Meningococci in Cooperation with Normal Adult Sera. Abstracts of the 16th Interscience Conference on Antimicrobial Agents and Chemotherapy. No. 179, 1976.
- 13. Smith, L. F., Lowell, G. H., MacDermott, R. P. Inhibition of Lymphocyte-Mediated Antibody-Dependent Killing of Group C Meningococci (Mgc) by Group C Mgc Polysaccharide. Abstracts of the 16th Interscience Conference on Antimicrobial Agents and Chemotherapy. No. 180, 1976.
- 14. Lowell, G. H., Smith, L. F., Nash, G. S., MacDermott, R. P. Null Lymphocyte-Mediated Antibody-dependent Cellular Cytotoxicity (ADCC) of Meningococci. Abstracts of the Annual Meeting of the American Society for Microbiology, p. 103, 1977.
- 15. Smith, L. F., Lowell, G. H., Bertram, M. A., MacDermott, R. P., Griffiss, J. M. IgG & IgA Dependent Mononuclear Cell-Mediated Killing of Meningococci. Abstracts of the Annual Meeting of the American Society for Microbiology, p. 102, 1977.
- 16. Yamada, K. K., Sadoff, J. C., Lowell, G. H. Effect of Pseudomonas aeruginosa Toxin A on Human Mononuclear Cells. Abstracts of the 77th Annual Meeting of the American Society for Microbiology, p. 33, 1977.
- 17. Cross, A. S. Diagnosis of Access Site Infections in Patients Undergoing Chronic Hemodialysis, American Society for Microbiology Annual Meeting, New Orleans, 1977.
- 18. Sidberry, H. D., Sadoff, J. C. Pyocin Sensitivity of Neisseria gonorrhoeae and its Potential as an Epidemiologic Tool. Inf. and Imm. 15:628-37, 1977.
- 19. Richardson, W. P. and Sadoff, J. C. Production of a Capsule by Neisseria gonorrhoeae. Inf. Imm. 15:663-64, 1977.
- 20. Bjorn, M. J., Vasil, M. L., Sadoff, J. C., Iglewski, B. H. Incidence of Exotoxin Produced by Pseudomonas Species. Inf. Imm. 16:362-66, 1977.

Table 1. Chemical Composition of Polysaccharide and LPS from  $\underline{P}$ .  $\underline{aeruginosa}$  Type 1

Component	Po	lysaccharide	LPS	
Carbohydrate		70 <sup>1</sup>	401	
Lipid		0.01	15	
Nucleic Acid		1.0	1.6	
Phosphate <sup>2</sup>		0.0	2.8	
Protein		0.0	4.3	
Water		21.67	ND3	
	TOTAL	92.68	63.7	

 $^{1}\mbox{Weight per cent}$   $^{2}\mbox{Phosphate determined on sample passed over a DEAE column in water to remove last trace of nucleic acid <math display="inline">^{3}\mbox{Not done}$ 

Table 2. Protection of Mice by Immunization with P. Aeruginosa Type 1 Polysaccharide

	nt (μgm) s given	#LD50 given	#Dead 72 hr Total	% Protected
100		30	4/10	60
50	Antigen from	30	4/10	60
25	solid culture	30	4/10	60
10		30	6/10	40
0.	5	10	7/8	12.5
0.	1	10	8/8	0
0.	05	10	8/8	0
100		66	2/9	77.8
25	Antigen from solid	66	4/9	55.5
10	culture	66	4/9	55.5
100	Antigen	66	3/9	66.7
50	from liquid	66	4/9	55.5
10	culture	66	4/9	55.5
500	Antigen from	82.5	4/9	55.5
250	solid culture	82.5	3/9	66.7

Mice were immunized by i.p. injection of amount of polysaccharide indicated in 0.1 ml saline and challenged 7 days later with an 18 hr agar culture of the organism suspended in saline to a predetermine optical density at 650 nm. 0.1 of challenge inoculum given.

Table 3. Rises in Antibody to Heterologous Lipopolysaccharide Antigens Following Vaccination with J-5 Organisms as Measured by Solid Phase Radioimmunoassay

	Pre	Post
J-5	.54 <sup>a</sup>	116.64
Re	.59	22.63
0111	.48	22.23
E. coli		
04	.08	.87
Serr.	.14	.25
Styph.	.13	.37
E. coli		
017	.27	2.74
Ps 1	.18	2.57
Ps 2	.47	7.64
Ps 3	.41	6.89
Ps 4	4.13	2.40
Ps 5	1.05	3.27
Ps 6	.61	9.63
Ps 7	.78	2.87

 $<sup>^{</sup>m a}$ Microorganisms of antibody/ml is a pooled sea from 5 rabbits prior to and following vaccination with heat killed J-5 organisms

J-5 J-5 mutant <u>E. coli</u> 0111

Re Re mutant Salmonella minnesota

0111 J-5 <u>E. coli</u> parent strain

E. coli 04 Smooth E. coli

Serr. Serratia marcescans clinical isolate

S. typhi Salmonella typhimurium

Ps 1-7 Pseudomonas aeruginosa immunotypes 1-7

Table 4 Rises in Antibody to Heterologous Lipopolysaccharide Antigens
Following Vaccination with J-5 Organisms as Measured by Solid
Phase Radioimmunoassay

	Pre	Post
J-5	.54	116.64
M978	.51	8.07
M35E	1.22	10.93
M981	1.61	15.29
M89I	1.08	2.36
M126	.98	9.04
M6275	1.32	4.15
M6155	.63	8.47
M992	3.20	14.91
M7889	.90	3.44

M978-M992 Prototype meningococcal LPS. Strains from groups B and C M7889 Prototype meningococcal LPS strain from group A.

Table 5 Comparison of Complement-Mediated and Cell-Mediated Killing of Group C Meningococci in Cooperation with Purified IgG

Amount of Csss bound	% Specific Kill	
(nanaograms)	ADCC	Ab + C'
32	74	95
16	70	86
8	65	82
4	52	78
2	26	5
1	18	7

Table 6 Comparison of Complement-Mediated and Cell-Mediated Killing of Group C Meningococci in Cooperation with Purified IgM

Amount of Csss bound	% Spe	cific Kill
(nanograms)	ADCC	Ab + C'
12.8	47	85
6.4	43	93
3.2	41	97
1.6	27	99
0.8	16	99
0.4	0	98
0.2	0	98

Table 7 Inability of IgA to Block IgG-Dependent Cell-Mediated Killing of Group C Meningococci

Csss	nt of bound ograms)	% Speci Kill (ADCC	
IgG	IgA	expt. #1	expt. #2
8	0	71	56
8	1	79	nd
8	2	80	nd
8	4	76	75
8	8	71	59
8	16	nd	54

Table 8. Comparison of Complement-Mediated and Cell-Mediated Killing of Group C Meningococci in Cooperation with Purified IgA

Amount of Csss bound	% Specific Kill		
(nanograms)	ADCC	Ab + C'	
9.6	52	0	
4.8	58	0	
2.4	60	0	
1.2	63	0	
0.6	50	0	
0.3	34	0	

Table 9. Summary Comparison of the Abilities of IgG, IgA and IgM to Kill Group C Meningococci in Cooperation with either Complement or Non-Adherent Mononuclear Cells

	Ab + Cells	Ab + C'
Amount of Csss bound (nanograms)	4.0 0.6	4.0 0.6
IgG	++ -	++ -
$I_{\mathbf{g}}M$	+ -	+++ +++
IgA	++ ++	

Table 10. Antibody-Dependent Cell-Mediated Killing of Meningococci in Cooperation with Purified Leucocyte Subpopulations

Cell Type	% Specific Kill ± SEM
Mononuclear cells (T,B,N.M)	65 ± 2 31 ± 5
Lymphocytes (T,B,N)	$43 \pm 14$ $31 \pm 6$
B cells	11 ± 4 18 ± 6
cells	6 ± 6 12 ± 6
ull (K) cells	67 ± 18 59 ± 8
onocytes	83 ± 10 53 ± 17
olymorphonuclear leucocytes	74 ± 1 69 ± 4

Table 11. IgG-Dependent Cell-Mediated Killing of Meningococci in Cooperation with Purified Leucocyte Subpopulations

	% Specific Kill ± SEM
Mononuclear cells	46 ± 7
Lymphocytes (T,B,N)	43 ± 7
B cells	16 ± 9
T cells	3 ± 2
Null (K) cells	48 ± 2
Monocytes	51 ± 5
Polymorphonuclear leucocytes	63 ± 1

Table 12. Cell-Mediated and Complement-Mediated Bactericidal Activity in Pre- and Post- Immunization Sera

	ADCC	C' + Ab			
PRE:					
Positive Sera (%)					
Total	19/20 (95%)	11/20 (55%)			
Exclusive	9/20 (45%)	1/20 ( 5%)			
Recip. of Geo. Mean Titre	86	25			
Titre Higher for (%)	15/20 (75%)	3/20 (15%)			
Mean Titre Difference	4.0 fold	4.6 fold			
POST:					
Positive Sera (%)					
Tota1	9/9 (100%)	9/9 (100%)			
Exclusive	0	0			
Recip. of Geo. Mean Titre	297	470			
Titre Higher for (%)	4/9 (44%)	5/9 (56%)			
Mean Titre Difference	3.6 fold	3.2 fold			
PRE vs. POST:					
Mean Titre Increase	5.6 fold	9.2 fold			

Table 13. Inhibitory Effects of Pili and Native Complex on Epithelial Cell Attachment

μg/ml	Organism		mg/ml	Orga	nism	
Pili 135	135 149	418	Nat. Com 135	135	149	418
0.45	84* 64	48	2	90	81	78
.22	73 49	39	1	86	43	-
.11	60 23	23	.50	75	28	59
.06	52 14	8	.25	72	26	43
.03	41 7	8	.12	73	29	26
.015	24 4	0	.06	59	22	17
.007	18 0	0	.03	61	25	0
.013	1		.015	42	19	0
.001	0		.007	28	19	3
.005	4					
Pili 149			NC139			
0.34	81 92	94	0.43	67	82	84
.17	73 88	84	0.21	47	67	47
.08	62 88	72	.11	46	57	11
.04	49 84	64	.06	14	56	2
.02	53 75	49	.03	14	47	0
.01	47 71	40	.015	3	37	0
.005	11 63	34	.007		32	
.002	10 63	15	.003		26	
.001	8 35	0				
.005	4 4	0	-			
Pili 418			NC418			
0.28	84 86	87	.33	29	71	64
.14	73 81	87	.16	23	47	15
.07	51 79	74	.08	10	45	4
.03	- 76	58	.04	0	23	Ö
.015	32 68	40	.02	0	8	4
.007	10 60	42	.01		0	
.0035	0 47	36	<u> </u>			
.0011	4 24	28				
.0001	13	17				
.0004	4	16	<u>-</u>			

 $<sup>\</sup>star$  % inhibition of epithelial cell attachment.

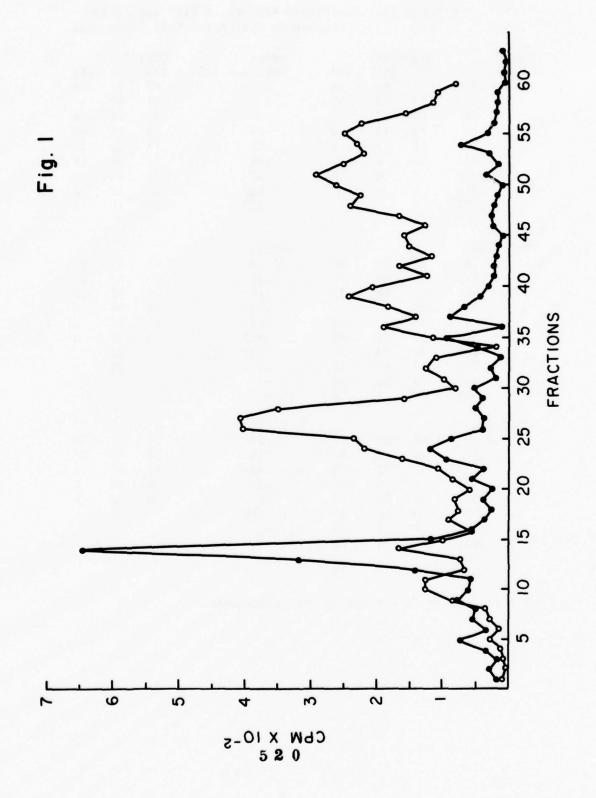
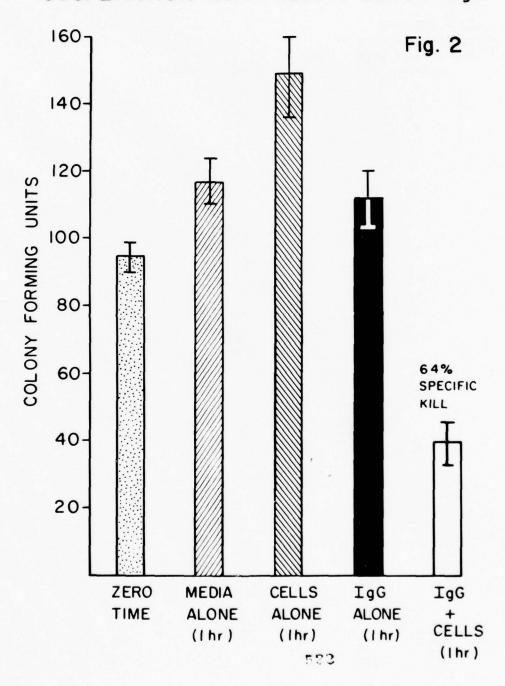


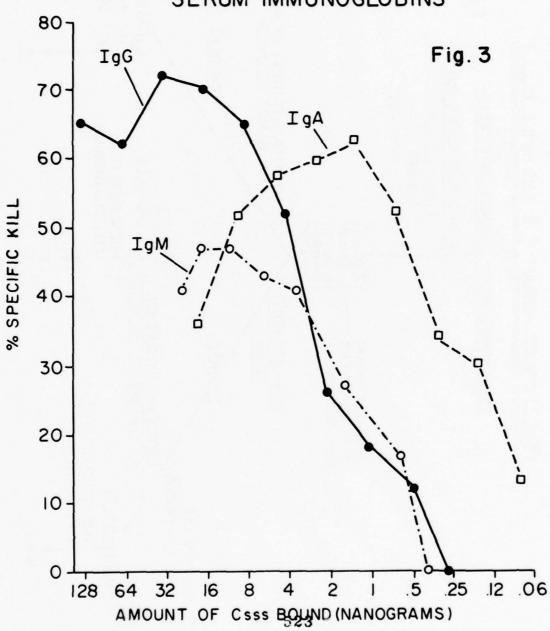
Figure 1 Reaction products of Pseudomonas Toxin A and Pseudomonas Toxin S.

Legend - SDS-PAGE CA insoluble product of  $C^{14}$  NAD + wheat germ - Toxin A  $C^{14}$  NAD + wheat germ + Toxin S

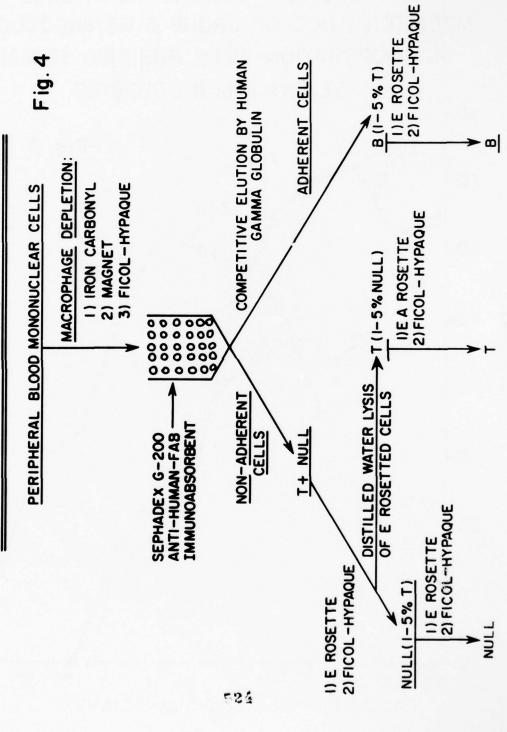
# ADCC OF GROUP C MENINGOCOCCI IN COOPERATION WITH HUMAN SERUM IgG

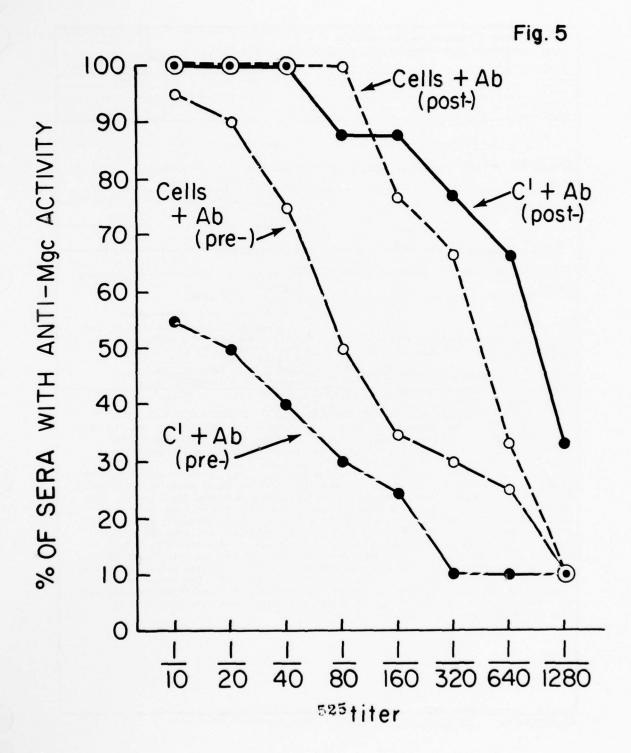


# NON-ADHERENT MONONUCLEAR CELL MEDIATED ADCC OF GROUP C MENINGOCOCCI IN COOPERATION WITH PURIFIED HUMAN SERUM IMMUNOGLOBINS



ISOLATION OF HUMAN T, B, AND NULL CELLS





DECEMBER AND TECHNOLOGY MORE INSTRUMENT			DA OB 6531		77 10 01		REPORT CONTROL SYMBOL			
RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY							R&E(AR)636			
3. DATE PREV SUMPRY	4. KIND OF SUMMARY	S. SUMMARY SCTY	S. WORK SECURITY	7. REGRA	DING	94 D	SO'N INSTR'N	SE SPECIFIC	DATA-	9. LEVEL OF SUM
76 10 01	D. Change	U	U	NA			NL	100	□ HO	A. WORK UNIT
10. NO./CODES:*	PROGRAM ELEMENT	PROJECT	NUMBER	TASK AREA NUMBER			WORK UNI	NUMBER	1	
& PRIMARY	61102A	3M161102	BS01	00 133						
b. CONTRIBUTING	CADDO 331/E									
XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX	CARDS 114F									
(U) Ecology	CHNOLOGICAL AREAS				_					
010100 Micr	objeless									
13. START DATE	ODIOTOGY	14. ESTIMATED COMP	PLETION DATE	IS FUND	HG A	GENCY		16. PERFORM	ANCE MET	нов
73 07		CONT		DA	- 1		1	C In-	-House	
17. CONTRACT/GRANT		CONT				S ESTIMAT	E & PROFESS	HONAL MAN YR		OS (In thousands)
A DATES/EFFECTIVE:	NA	EXPIRATION:				EDING				
& NUMBER:*				FISCAL		77		2 179		79
C TYPE:		4 AMOUNT:		YEAR	CURR					0
& KIND OF AWARD:		f. CUM. AMT.				78		2	180	
19. RESPONSIBLE DOD	DRGANIZATION			20. PERF	ORMII	G ORGANI	EATION			
MAME: Walter	Reed Army Ins	titute of	Research	WWE:	ter	Rood	Army In	ctituto	of D	a a a a wah
		ereuce or	nesearen	Walter Reed Army Institute of Research Div. of CD&I Washington, DC 20012						
ADDRESS:* Washin	gton, DC 200	12								
	g, 20 200									
				PRINCIPAL INVESTIGATOR (Pumloh SEAN II U.S. Academic Institution)						
RESPONSIBLE INDIVIDU				NAME: Cavanaugh, Dan C., Ph.D., COL, MSC						
MAME: Rapmund, Garrison, COL, MC			TELEPHONE 202-576-5176 or 5110							
TELEPHONE: 202-576-3551			SOCIAL SECURITY ACCOUNT NUMBER:							
Foreign intelligence not considered.			ASSOCIATE INVESTIGATORS  NAME: Williams, James E., MAJ, MSC							
			wave Harrison Daniel N Ph D							
21. REVENUE (Procedo EACH with 300mily Closelfication Code) (U) Yersinia pestis; (U) plague; (U) vaccines;										
(U) immuniz	ation; (U) se	rological	tersinia pe	Cene	, (	o) bra	igue; (U	) vaccir	ies;	
	TIVE. 24 APPROACH, 28.						est of each wife :	Security Classific	cetten Code	.,
23. (U) Det	ermine the fa	actors infl	uencing ou	tbrea	ks	of pla	ague inf	ection a	and ti	he most
appropriate	e methods to	revent the	infection	of t	roo	ps en	gaged in	field o	opera	tion.
24. (U) Usi	ing standard m	nethods, sp	ecimens an	d ser	a f	rom h	umans an	d anima	ls ar	e
tested for	the presence	of Y. pest	is and F-1	anti	bod	y to	Y. pesti	s.		
25. (U) 76	10 - 77 09	Strains of	Y. pestis	rece	ntl	y iso	lated fr	om natu	ral f	oci of
plague were	e examined for	r virulence	determina	nts,	abe	rrant	protein	conten	t, an	d anti-
biotic sens	sitivities. S	Strains exa	mined (43)	from	So	uth V	ietnam,	Java, a	nd the	e USA
produced Fr	raction 1 anti	igen, VW an	tigens, pe	stici	ns,	pigm	entation	and the	ey we	re
	to streptomyc									
encountered			riophages							
tools for t	the study of o									
	in progress.									
	nereafter chal									
deficient Y. pestis failed to prove susceptible to Y. pestis. Resolution of the question of the susceptibility of primates to chronic plague awaits experiments with										
more suitable monkeys. For technical report see Walter Reed Army Institute of										
Research Annual Report, 1 Jul 76 - 30 Sep 77.										
nesses and report, I sai to see the										
	728									

Project 3M161102BS01 BASIC RESEARCH ON MILITARY DISEASES

Work Unit 133 Ecology of Plague

Investigators.

Principal: COL Dan C. Cavanaugh, MSC

Associates: MAJ James E. Williams, MSC; CPT Lyman Roberts, MSC;

Daniel N. Harrison, Ph.D.; Peter Gemski, Jr., Ph.D.; LTC Paul K. Hildebrandt, VC; SP4 Michael Fortier;

SP5 Michael Richards

# Description

To determine the factors influencing outbreaks of plague infection and the most appropriate methods to prevent the infection of troops engaged in field operation.

### Progress

The plague outbreaks of 1975 and 1976 in the United States provided interesting epidemiological data for many of the plague cases that occurred. Prompted by the promise of fresh insights into the as yet unresolved problem of persistent endemic plague, and the fact that numerous DOD installations are in or near natural plague foci, an examination of trends in the incidence of plague in the United States was undertaken. As in previous years, plague strains recovered from patients have been studied for virulence determinants and sensitivity to antibiotic drugs. Although all plague strains recovered from patients in the United States over the past few years have been of typical phenotype and sensitive to streptomycin and tetracycline, a number of aberrant plague bacteria have been found in the tissues of fleas, rodents, and man (22). Such observations, reinforced by laboratory experiments on chronic plague in rats (21) have suggested that atypical phenotypes of the plague bacillus are not uncommon in nature and have stimulated research in the period covered by this report to: (1) test the abilities of various vaccines and drugs to prevent cryptic plague in rats and primates; (2) develop and test methodologies directly applicable to studies of Y. pestis phenotypes and chronic disease; (3) determine the frequency of occurrence of atypical Y. pestis in naturally infected fleas and mammals; and (4) continue the studies on  $\underline{Y}$ . pestis sensitivity to various bacteriophages. In addition, new studies were undertaken to test vaccines for protection against plague and to improve diagnostic reagents and procedures for the diagnosis and surveillance of plague infection. These matters form the basis of this report.

I. An analysis of plague in the United States.

The persistence of natural plague foci and the likelihood of increased human contact with the plague bacillus with development schemes or other activities has been recognized internationally (23) and is supported by

reports from widely distributed natural foci in the western United States (1,2). Table 1 presents the data on cases of plague resulting from probable contact with wild rodents or their fleas that have occurred in the United States since 1920; the decade in which sylvatic plague was first recognized as a distinct entity. By definition, sylvatic or wild plague is a rural disease. Although the rural populations (3) of the nine states listed in Table 1 are rather constant, the average number of cases per year shows a rise in incidence. These data may be somewhat conservative as several plague cases are known to have been misdiagnosed as tularemia (18). Young children appear to be principal victims (Table 2), with exposure in the vicinity of the home. The geographic distribution of the cases (Figure 1) shows two pronounced natural foci: one along the Pacific Coast and associated inland mountain ranges; one in the general area of the Rocky Mountains. While all regions do not demonstrate the same degree of activity, sporadic cases occurring over the years attest to the persistence of the disease in many of the areas shown. To provide a more precise identification of areas of potential significance to DOD, the coordinate grid utilized in Figure 1 is based upon Map 40-DMATC, Series 8205 of 2-76, "Major Army, Navy and Air Force Installations in the U.S."

The persistence of plague in natural foci in the United States is impressive. Current studies at WRAIR provide some insite into possible mechanisms for this phenomena. Antibodies to the specific Fl capsular antigen of Y. pestis are detectable in the peripheral blood of human beings, rodents, and certain other mammals shortly after infection. Fl antibodies can be demonstrated in the sera of animals collected in plague foci, and serum surveys have been widely used for plague detection and surveillance. Maternal Fl antibody is also present in the sera of young R. norvegicus and other rodents born to infected or vaccinated dams. Presence of F1 antibody as measured by the indirect hemagglutination test reflects an immune state which results from (1) vaccination with either living attenuated or killed plague vaccines, or (2) injections of potent plague antisera. Rattus norvegicus with titers in excess of 1:64 are usually highly resistant to acute disease. Unfortunately, Y. pestis are not always eliminated from the tissues of all seemingly immune rats, and the disease occasionally persists in chronic form. Walled-off buboes, containing a spectrum of varying Y. pestis phenotypes, continue to enlarge (Figure 2) and may result in the eventual death of the animal. Death in laboratory rats usually occurs when buboes rupture internally and release their highly toxic and infectious contents into the system of the animal. This condition is noteworthy (Table 3) because  $\underline{Y}$ . pestis of fully virulent phenotype (F1<sup>+</sup>:VW<sup>+</sup>:P<sup>+</sup>:PCF<sup>+</sup>) may persist in the tissues of vaccinated animals up to 18 months following challenge infections, and virulent variants of Y. pestis (FI-: VW+: P+: PCF+), selected in vivo in the presence of F1 antibody, have been found 24 months post challenge. The

mechanisms for the survival of  $\underline{Y}$ .  $\underline{pestis}$  and for the selection of variant bacilli  $\underline{in}$   $\underline{vivo}$  in immunized rats is not clear, but the data suggest processes which serve to maintain the presence of the disease in natural foci. However, the presence of atypical  $\underline{Y}$ .  $\underline{pestis}$  in nature is of concern.

Several relevant observations appear justified based on experience in R.V.N. and the above analysis. Plague in natural foci is persistent. Vast tracts of land in the western United States are utilized for various DOD purposes. Obviously, residents in natural plague foci are at some risk and 9 of the cases shown in Table 1 were active or retired military personnel or their dependents. Several DOD installations have reported the presence of infected rodents during the present decade. Seasonal distribution of the cases (Figure 3) shows that the threat is present throughout the year. The distinct peaks are characteristic of flea-borne plague, while "off-season" cases may illustrate the potential dangers of handling dead or infected animals (17,19). Actual contact with natural foci, however, may be unnecessary, as in some instances, the infection involves the respiratory system, and the patient transmits plague bacilli via aerosol or fomites to his contacts elsewhere. Treatment with streptomycin, tetracyclines, or chloramphenicol has greatly reduced plague mortality. However, as noted in Vietnam, these drugs do not always result in the complete elimination of Y. pestis from walled-off lesions: virulent Y. pestis have been isolated from some patients who have been under treatment for prolonged periods of time (13). Residual bacilli could result in a chronic infection. Bubonic plague occurs in all degrees of severity. In some circumstances, patients first seen late in the course of the disease may show frank bacteremia (Figure 4), a grave prognostic sign. In some patients, disseminated intravascular coagulation may present a problem, in extreme cases leading to purpura (Figure 5) and frank gangrene resulting in loss of extremities (4,16). A guarded prognosis is always advisable, as some patients may succumb even when apparently recovering. Reports of chronic, relapsing, latent meningeal plague (15) and recurrent plague infection without accompanying serological response (5) are suggestive of either chronic infections or infections with aberrant phenotype (F1-:VW+:P+:PCF+). Such variants have in the past been encountered (22).

# II. Investigations of chronic plague in rats and monkeys.

As stated previously, chronic plague occurs with some frequency in laboratory rats, the actual frequencies will be known when several long-term laboratory experiments are completed. Of particular interest, are experiments with non-immune rats that were infected with classical plague bacilli and subsequently treated with streptomycin or tetracycline to determine if either or both drugs prevent chronic infections. To date, cryptic plague has not been found among treated rats.

Work on cryptic plague reported previously (WRAIR Annual Report for 1 Jul 74 - 30 Jun 75) employed either the encapsulated virulent Indian strain 195/P of Y. pestis or nonencapsulated variants derived from 195/P.

Consequently, it has not been certain if chronic, latent or cryptic plague is a peculiar manifestation that follows infection only with 195/P. Therefore, experiments were undertaken with additional strains of fully virulent  $\underline{Y}$ . pestis recently isolated from plague patients in Vietnam and the USA. Challenges were done, as in past experiments, in a manner that simulated infection via fleabite. The rats challenged included unvaccinated controls and animals that had received plague vaccine USP (1 ml IP and 0.5 ml booster 3 weeks later and a month before challenge). To date, four instances of chronic plague have been detected in rats infected with plague strains from Vietnam and four with plague strains from the United States (Table 4), establishing that long-term plague infections in certain Rattus norvegicus are typical complications that arise after infection with  $\underline{Y}$ . pestis.

Experiments designed to determine if cryptic plague occurs in Rhesus monkeys vaccinated with the presently approved Army plague vaccine USP have been complete. None of 23 monkeys studied were found to harbor plague infections 18 months after challenge (Table 5) or demonstrate suspicious pathological indications of chronic disease. Buboes noted in several monkeys which persisted for a month after challenge had resolved completely. The high resistance of Rhesus monkeys to bubonic plague as noted by other investigators (6,14) was demonstrated dramatically. Although at least 75% of the monkeys in the 195/P group responded serologically to infection (Table 5), only a single death occurred. Also, none of the monkeys challenged with the nonencapsulated plague strain CPS-2a died. Serological criteria to establish infection were not available, since the IHA and CF tests currently employed at the WRAIR specifically measure antibodies to the capsular F1 antigen of Y. pestis.

In view of the naturally high resistance of Rhesus monkeys to plague, conclusions about the probability of cryptic plague in man from experiments with this monkey are impossible. More definitive evidence for or against such possibilities must await experiments in more susceptible primates.

### III. Discontinuous polyacrylamide gel electrophoresis.

Discontinuous polyacrylamide gel electrophoresis suitable for epidemiological investigations of plague (12) also appears useful for investigations of: (1) atypical plague strains to determine antigenic differences from typical  $\underline{Y}$ . pestis, (2) immune responses to plague infection to help establish how chronic infections differ from typical plague, (3) vaccines or diagnostic reagents produced by various methods or at different times, and (4) the bacteriology of  $\underline{Y}$ . pestis. In the period covered by this report, a system of discontinuous gel electrophoresis (as established by Dr. Joel Dalrymple, WRAIR) was tested, and is presently being utilized in the areas of research mentioned above.

In brief, the system utilizes polyacrylamide-SDS slab gels in a Bio-Rad Model 220 slab unit. Extracts of acetone powders of  $\underline{Y}$ . pestis or other sample materials are applied to 3.6% polyacrylamide stacking gels of pH 6.7 over 10% polyacrylamide-SDS separation gels of pH 9.3. Replicate slab gels are electrophoresed at a constant current of 40 ma for approximately 5 hours. The gels are then stained with Commassie Blue stain, destained in methanol-acetic acid solutions and photographed. Gels are scanned with a densitometer to quantify the patterns obtained by electrophoresis, and the data obtained thereby are subjected to analysis of variance, using a randomized complete-block design, to statistically establish differences among the sample materials under study.

The technique has now been used to advantage in an examination of plague strains isolated between 1972 and 1974 in the Bojolali Regency of Central Java. Strains from this mountainous region demonstrate a heavy band (#3 in our system) not always present in plague strains that have been isolated in nearby lowland areas. Six plague strains isolated in 1972 at Bojolali had band #3, as did a strain obtained in 1974. However, a plague strain isolated late in 1973 was distinctly different in that band #3 was absent (Figure 6). Either  $\underline{Y}$ . pestis lacking band #3 do occur with some frequency in the mountains of Bojolali, in which case this constitutes a first observation, or the Y. pestis strain isolated in 1973 was imported from an active but unrecognized plague focus in the lowlands. In either case, the epidemiology and ecology of plague in Central Java appears to be somewhat more complicated than expected. New isolates in the United States and elsewhere will be examined by this electrophoresis technique to determine likely geographic sources of infection.

An investigation designed to compare the serological responses in rats infected with aberrant  $\underline{Y}$ .  $\underline{pestis}$  with those in rats infected with plague bacilli of classical or aberrant phenotype has been started. Pilot experiments to obtain baseline information for infection with typical  $\underline{Y}$ .  $\underline{pestis}$  strain 195/P have revealed that plasmas collected pre-infection and from moribund rats give amazingly different electrophoretic profiles. Antibody patterns will be studied in the coming year.

Discontinuous gel electrophoresis was also most useful in comparisons of fraction-1 (F1) preparations produced in various nations for diagnostic tests to detect plague antibody (Table 6).

#### IV. Bacteriological studies.

Previous studies (Annual Report, 1976) demonstrated the action of the standard  $\underline{Y}$ .  $\underline{pestis}$  "H" and the  $\underline{Y}$ .  $\underline{pseudotuberculosis}$  "IV" phages on various strains of  $\underline{Yersinia}$  at different temperatures. The studies showed incomplete lysis in a large number of strains. Further, temperature was a definite factor involved in the specificity of the lytic reaction.

This report concerns the activity of rough specific bacteriophages of the <u>Enterobacteriaceae</u> on the plague bacillus. This extended test system may provide valuable data for the development of an epidemiologically sound method of fingerprinting and/or typing  $\underline{Y}$ . pestis.

Several studies have been conducted on the nature of smooth (S) and rough (R) forms of  $\underline{Y}$ . pestis (7,8,9,10,11). The general conclusion is that the plague bacillus exists worldwide as a heterologous mixture of S and R variants, with the S forms in predominance. Since the above investigators were restricted to visual observation only: (1) the purity of a given R strain could not be guaranteed; (2) attempts to distinguish S and R antigenic components were inconclusive; (3)  $R^{-}$  S dissociations could be possibly, a result of environment; (4) virulence could not be based upon the S and R classifications then available. There seems to be a question as to whether true R variants exist in  $\underline{Y}$ . pestis as in other Enterobacteriaceae.

By contrast, the R forms of <u>Salmonellae</u> are well characterized. Of special interest are the rough specific bacteriophages of <u>S. typhimurium</u> and other enterics. Not only can the lack of the O (somatic) antigen be detected by this unique series of phages but mutants affected in specific sugars, enzymes, etc. in their LPS synthesis can be pinpointed. The application of these phages to  $\underline{Y}$ , pestis could possibly resolve some of the difficulties mentioned above. Also, the phage action could determine whether strains of  $\underline{Y}$ , pestis can be divided into specific groups leading to a practical and useful typing scheme.

Screening studies. Seventy-five plague strains (31 morphologically R; 34, a mixture of S and R, and 10 morphologically S) were tested for lytic sensitivity to 6 rough specific enteric bacteriophages (Br $_2$ , Ffm, C $_{21}$ , 6SR, ES18, and BR60) (20). The cultures were streaked on blood agar or Brain Heart Infusion agar plates (BHIA) and incubated at 28°C for 48 hours. They were then inoculated into 3 sets of peptone broths and were incubated at 22°C, 28°C, and 37°C, respectively for 24 hours. The plague strains were then swabbed onto BHIA plates and the test phages with titers of  $10^8$  plaque forming units (PFU) per ml were applied using 1 ml glass syringes fitted with 25 gauge needles. The results of the sensitivity patterns of the preliminary tests are shown in Table 7.

Table 7 shows that phage sensitivity was evident, especially among phages 6SR and BR60 at  $22^{\circ}\text{C}$ . Overall, the heterogeneous nature of  $\underline{Y}$ . pestis as reported by Eisler was confirmed by the inconsistent patterns of sensitivity. For example, the visually S strains appeared to be as sensitive as the R ones. A further analysis (Table 8) shows that most of the phage action on the S strains was marked by scattered individual plaques: thus, only a small percentage of the whole  $\underline{Y}$ . pestis population

was sensitive to lysis. Occasional mutants deficient in some form of LPS synthesis may have been pinpointed by the bacteriophages. Similarly, morphologically R strains may not be entirely R by chemical analysis: their colonial appearance might be due to environmental influences. As suggested by the poor phage receptivity in the S-R class, this group may consist of more uniformly S cells than either the R or S classes. It is permature to draw conclusions but the development of an experimental system that selects for R variants may lead to consistent antigenic differentiation of  $\underline{Y}$ .  $\underline{pestis}$  cells by specific bacteriophages.

Interaction of the enteric phages and the plague bacillus. The high incidence of variability shown in Table 7 brought into focus the questions of the fate of the enteric phages in  $\underline{Y}$ . pestis and the possibility of broadening their host ranges.

The morphologically R  $\underline{Y}$ .  $\underline{pestis}$  strain DF1 (deficient in Fl production) was selected as the host for further study. Six BHIA plates were swabbed with 24 hr broth cultures of DF1 and enteric R phages were applied separately to each plate. They were incubated at the most favorable temperature (22°C) for 24 hours. Phages  $Br_2$ , 6SR, and BR60 rapidly produced large lytic zones. Ffm formed plaques after 5 days. However, both  $C_{21}$  and ES18 failed to produce plaques on DF1 throughout a 10 day observation period. Plaques were aseptically cut from the agar and placed in sterile broths. The phages were cultivated, reisolated from the plaques, and again propagated on DF1 to a titer of  $10^6$  PFU/ml. The efficiency of plating (EOP) for the 4 passaged phages grown either on DF1 or  $\underline{S}$ .  $\underline{typhimurium}$  are shown in Tables 9 and 10.

No significant restrictions by <u>S</u>. typhimurium were evident (EOP $^{\pm}$ 1) with Br $_2$ , 6SR, and BR60 phages grown on DF1. However, Ffm propagated on DF1 was restricted by its original host <u>S</u>. typhimurium (the universal host of these rough specific enteric phages). This may be true only for DF1 since <u>S</u>. typhimurium did not restrict Ffm when it was grown on another R plague strain (EXU $^{\sharp}$ 1).

None of the phages grown on  $\underline{S}$ . typhimurium were restricted by DF1 at  $22^{\circ}C$  but there is some evidence of restriction by DF1 at  $28^{\circ}C$ . Therefore, many of the token and turbid plaques shown in Table 8 probably reflect restrictions of  $\underline{S}$ . typhimurium propagated phages. Further support for this conclusion came from the phages grown on DF1 (Table 9), which when applied to plague cells, caused complete lysis.

Hosts for the propagation of rough-specific enteric bacteriophages in  $\underline{Y}$ .  $\underline{pestis}$  should be selected with some judgment. One rough-specific phage,  $\underline{Ffm}$ , became greatly reduced in specificity for its original host,  $\underline{S}$ .  $\underline{typhimurium}$  following a single passage in the  $\underline{Y}$ .  $\underline{pestis}$  R strain DF1.

However, as a result of this passage, Ffm became more specific for the R characters present in the  $\underline{Y}$ .  $\underline{pestis}$  strain. Further studies with rough-specific phages should prove valuable in detecting other factors involved in the cell wall complex of  $\underline{Y}$ .  $\underline{pestis}$ . The above preliminary studies are most promising; the interesting finding of the adaptation of an R-specific phage to an R strain of  $\underline{Y}$ .  $\underline{pestis}$  could be significant in developing a useful and practical typing scheme.

### V. Killed plague vaccines.

The USP vaccine approved for use by the Army is prepared from the highly virulent strain 195/P and special laboratory facilities are required for its production. At present, there is only one source producing the vaccine. In an emergency or catastrophic situation, it would be extremely difficult to rapidly replace or supplement this source of supply. Therefore, alternatives have been considered. Experiments were conducted with several experimental lots of killed vaccine prepared with avirulent, encapsulated strains of the plague bacillus (ATCC 11953 and EV76S) by the Department of Biologics Research, WRAIR. Vaccines were prepared by methods used for the approved USP vaccine in compliance with FDA regulations for IND. Three standard potency assays were performed to compare the experimental vaccines with various lots of USP vaccine, and, without exception, the experimental vaccines were of greater potency in mice than lots of USP vaccine (Table 11). Thus, killed plague vaccines prepared from encapsulated but avirulent Y. pestis are promising and probably can be developed to equal or exceed in efficacy the currently used USP vaccine. If required, the production of such a vaccine could be effected with fewer technical difficulties, since by using avirulent rather than virulent organisms in manufacturer large class 3 facilities would not be required.

#### VI. Diagnostic reagents.

In the past year, an effort has been made, in collaboration with WHO to standardize F1 antigen preparations used in serological tests for plague antibody, and to compare indirect-hemagglutination (IHA) test procedures now in use. F1 antigen preparations from the USA, USSR, and South Africa were tested concurrently (Table 6). The electrophoretic migrations of these substances by discontinuous polyacrylamdide-SDS gel electrophoresis were identical, and all gave essentially equal results in CF and IHA test systems. Furthermore, all F1 antigens demonstrated mouse 50% protective doses ranging within 1-10  $\mu g$  F1 per mouse. No consistent or significant differences were found among the F1 preparations. Although additional testing is in progress, information gathered so far indicates that the F1 antigens produced in the USA, the USSR, and South Africa are identical, and results of serological tests employing these preparations

of Fl should measure the same antibodies. To this conclusion, a comparison of IHA test procedures employed in the USA and the USSR added credence. Diagnostic test procedures and materials from the USSR were tested side-by-side with the system used at the WRAIR, and a freeze-dried control serum of high stability was sent to the CDC plague laboratory in Fort Collins, Colorado, for testing. Results showed that the test materials and procedures employed at WRAIR, CDC and USSR, are essentially identical. Thus, serum titers of IHA antibody reported by these laboratories are directly comparable.

Attempts to utilize other antigens of  $\underline{Y}$ . pestis for diagnostic reagents in serological tests to detect aberrant plague infections, have continued. Polyside preparations, utilized by French investigators (Institute Pasteur, Paris), have been prepared for this purpose.

#### VII. Miscellaneous studies.

In cooperation with the CDC Plague Branch, Fort Collins, Colorado, an investigation is underway to detect atypical  $\underline{Y}$ . pestis in naturally infected fleas and mammalian hosts. Two objectives are being sought: (1) to develop improved survey methods for the isolation of variant  $\underline{Y}$ . pestis, and (2) to obtain further information on frequencies of occurrence for the atypical  $\underline{Y}$ . pestis phenotypes that occur in the western United States. Plague strains isolated from new cases are also under investigation to extend recent work on virulence determinants and antibiotic sensitivities.

#### VIII. Conclusions and recommendations.

Plague persists in essentially permanent natural foci in the western United States. The disease may be contracted "year round" through either contact with infective fleas or infected animals. Laboratory investigations indicate mechanisms for persistence in nature and, also forecast atypical situations that could pose problems in diagnosis, vaccination, and therapy. Atypical plague bacilli, isolated from natural sources, provide some confirmation that the above laboratory observations do occur in nature. Examinations of  $\underline{Y}$ . Pestis of classical and aberrant phenotype by standard laboratory tests including protein electrophoresis and the rough-specific enteric bacteriophages promise to provide a better understanding of epidemiological patterns in nature and some insight into the unknown areas of the pathogenesis of the disease.

The persistence of the disease; problems with diagnoses and therapy; failure of vaccination to prevent chronic infection of  $\underline{R}$ . norvegicus; and an unpredictable potential for man-to-man aerogenic infection justifies continued study and surveillance.

Table 1. Plague cases of probable sylvatic origin reported in the United States: 1920-1976.

				cade				%
	20-29	30-39	40-49	50-59	60-69	70-76	Cases	Tota
Arizona				1	3	9	13	10
California	13	7	5	3	1	6	35	26
Colorado				1	3	2	6	4
Idaho			1		1		2	1
Nevada		1					1	1
New Mexico			3	4	21	40	68	51
Oregon		1				2	3	2
Texas					1		1	1
Utah		2			1	2	5	4
Cases	13	11	9	9	31	61	134*	
% Total	10	8	7	7	23	45		
Cases/yr	1.3	1.1	0.9	0.9	3.1	8.7		
Rural population (thousands)	6247	7150	7939	7432	6981	6553		

 $<sup>\</sup>star$  Excludes laboratory infections and urban plague.

Table 2. Age and sex distribution of sylvatic plague cases reported in the United States: 1920-1976.

Age	Male	Female	Total	% Total
0-4	4	9	13	11
5-9	11	12	23	19
10-14	17	13	30	25
15-19	13	2	15	12
20-24	5	3	8	7
25-29	5	2	7	6
30-34	2	3	5	4
35-39	6	1	7	6
40-44	-	-	<u>-</u>	-
45-50	2	2	4	3
> 50	6	3	9	7
Total	71	50	121	
% of Total	59	41		100

Table 3. Persistence of plague infection in some <u>Rattus norvegicus</u> surviving challenge with classical plague organisms.

Immunization before challenge	Isolation o	f <u>Yersinia</u> pestis
	Phenotype	Months post-challenge
Living avirulent plague vaccine (EV)	F1-:VW+:P+:PCF+	18
Killed plague vaccine (USP)	F1+:VW+:P+:PCF+	17
Anti-plague serum (polyvalent)	F1+: VW+: P+: PCF+	11
	F1-: VW+: P+: PCF+	24

Latent plague in vaccinated rats after infection with  $\underline{Y}$ .  $\underline{pestis}$  strains from recent human plague cases. Table 4.

Source of	Strain	Challenge*		Observat	Observations of latent plague
Y. pestis		Number Y. pestis Mouse LD <sub>50</sub>	Mouse LD <sub>50</sub>	No. rats	Months post-challenge
Human cases of	74-132D	5,100	8	1	12
Vietnam, 1974	74-135B	4,800	16	1	15
	74-135C	000*9	26	2	13; 16
Human cases with	762575	6,200	37	1	16
preumonia;	BAC001884	4,200	27	3	13; 15; 16
Arizona, U.S.A., 1975					

 $\underline{Y}$ . pestis were incubated at 25°C and inoculated subcutaneously in order to obtain an inoculum resembling that derived from fleabite.

All rats had been vaccinated with killed U.S.P. vaccine (Cutter Laboratories) prior to challenge, except the rat that died 13 months after infection with strain 74-135C. This was an unvaccinated control rat that survived challenge. \*

Table 5. Experiments to detect latent plague infection in Rhesus monkeys vaccinated with plague vaccine U.S.P.

Challenge	Monkey No.		IHA Titer		Percent +	Plague infections
		Prechallenge	1-month post	18-months post	Infected	18-months post
29 400 v 301 v 3 7	**076	1	102%	32		
4.3 A 10 1. pestis	** 607		1054	75		
Strain 195/P (F1 <sup>+</sup> )	285	,	•	1		
(2.5 X 10 <sup>5</sup> mouse LD <sub>e,0</sub> )	260	16		•		
	271	16	79	99		
	284	16	(died 6 days	(died 6 days after challenge)		
	281	32	16	99		
	171	32	>8192	512		
	103	32	512	16		
	263	79	512	7		
	270	79	1024	1024		
	859	79	49	79		
	255	128	1024	512		
	278	128	2048	512		
	816	128	>8192	1024		
	835	128	>8192	512		
	273	2048	>8192	8192		
					75%(12/16)	75%(12/16) None in 15 monkeys
4.4 X 10 <sup>6</sup> Y. pestis	258		•			
Strain CPS-2a (FI")	841		,	•		
(5.1 X 10 <sup>5</sup> mouse LD <sub>sO</sub> )	265	91	,			
20	266	16	,			
	261	79	16	60		
	272	99	7	4		
	277	79	80	80		
	298	99	,			
					11	None in 8 monkeys

Monkeys with <sup>2</sup> 4-fold increases in Fl antibody plus dead monkcy divided by total. Obviously, serological indications of infection with the Fl<sup>-</sup> strain were not detectable, and percent infected could not be calculated. Unvaccinated control.

\*

Table 6. Comparison of Fl antigens from the U.S.A., U.S.S.R. (WHO) and South Africa.

Control serior date   For band obtained by   Optimal   Control serior   Control serior   Concentration (ug F1)   Gose (ug F1)	Fl antigen	Densitomter readings	CF test in microtiter	microtiter	IHA test in microtiter	50% protective
84 ± 11.7     0.4     1:64     0.0005       101 ± 10.6     0.4     1:64     0.001       98 ± 14.1     0.4     1:64     0.0005       104 ± 10.6     0.4     1:128     0.001       6     83 ± 11.3     0.4     1:64     0.001	(preparation date)	for band obtained by disc gel electrophoresis of 5 $\mu$ g Fl ( $\bar{X}$ $\pm$ SD)		Control serum titer at optimal conc.	Concentration (µg F1) required to inhibit 2 units of antibody	dose (µg Fl) for mice*
$98 \stackrel{\pm}{=} 14.1$ $0.4$ $1.64$ $0.001$ $0.4$ $1.64$ $0.0005$ $104 \stackrel{\pm}{=} 10.6$ $0.4$ $1.128$ $0.001$ $0.4$ $0.4$ $0.4$ $0.001$	** Baker Lot 3 (Dec 69)	84 ± 11.7	0.4	1:64	0.0005	4.2
98 ± 14.1 0.4 1:64 0.0005  104 ± 10.6 0.4 1:128 0.001  83 ± 11.3 0.4 1:64 0.001	WRAIR Lot 4 (Jul 71)	101 ± 10.6	9.0	1:64	0.001	1.9
$104 \pm 10.6$ 0.4 1:128 0.001 83 ± 11.3 0.4 1:64 0.001	U.S.S.R. Lot 8 (Sep 76)	98 ± 14.1	0.4	1:64	0.0005	5.2
83 ± 11.3 0.4 1:64 0.001	U.S.S.R. Lot 9 (Sep 76)	104 ± 10.6	0.4	1:128	0.001	7.9
	S. Afr. Lot 4-76 (Apr 76)	83 ± 11.3	7.0	1:64	0.001	4.2

\* Challenged with 10,000 mouse  $L_{\rm D_{\rm SO}}$  of virulent Y. pestis strain 195/P

\*\* Antigen preparation of documented purity (Baker et al., J. Immunol. 68: 131, 1952).

Table 7. Sensitivity patterns of Yersinia pestis at different temperatures to the rough-specific phages of various Enterobacteriaceae.

		_							Rough	phag	Rough phages tested	sted							
			Br,			Ffm			C21			6SR			ES18			BR60	
Colonial	Colonial No. strains		,						Tempe	ratur	Temperature (°C)	_							
morphology tested		22	22 28 37 22 28 37 22 28 37 22 28 37 22 28 37 22 28 37	37	22	28	37	22	28	37	22	28	37	22	28	37	22	28	37
Rough	31	*11	11* 19 4 20 20 5 17 24 9 93 36 20 14 8 13 86 52	4	20	20	2	11	54	6	93	36	20	14	80	13	98		20
Semi-rough	34	6	9	0	٣	3 3 0	0	3	10	0	85	3 10 0 85 10 0 0 3 0 79 53 61	0	0	3	0	62	53	61
Smooth	10	п	57	57 0 11 38 20 22 57 20 100 38 11 0 22 0 100 72 55	=	38	20	22	57	20	100	38	11	0	22	0	100	72	55

\* The number denotes the percentage of strains tested that showed token, partial or complete lytic activity.

Summary of lytic patterns observed from testing rough-specific enteric phages (Br $_2$ :FFM: C21:6SR:ES18:BR60) on smooth or rough strains of  $\underline{\rm Y}$ .  $\underline{\rm pestis}$ Table 8.

Y. pestis colonial form Rough Semi-rough	Number strains tested 31	Complete confluent lysis 6.7	Pattern observed (per cent) Partial Token lysis lysis (turbid)  1.3 21 2.3 15	(per cent) Token, lysis 21 21	No 1ysis 71 81	
Smooth	10	2.4	60.	26.5	71	

\* Small discrete plaques resembling those resulting from either the action of a single phage unit or the presence of 1 sensitive cell in the test system.

Table 9. Tirers and efficiency of plating  $^{\star}$  of rough-specific enteric phages grown on DF1 at  $22^{\rm 0}{\rm C}$  and  $28^{\rm 0}{\rm C}$ 

	S. typhimurium	EOP	1.1	<1.6 X 10 <sup>-6</sup>	0.5	0.83
Host 28°C	S. typh	Titer	2.5 x 10 <sup>6</sup>	7	5 x 10 <sup>4</sup>	1 x 10 <sup>6</sup>
H		EOP	П	1	1	1
	DE1	Titer	1.5 x 10 <sup>6</sup>	1.6 X 10°	1 x 10 <sup>5</sup>	1.2 x 10 <sup>6</sup>
	nurium	EOP	0.7	<3 x 10 <sup>-6</sup>	4.0	0.76
Host 22°C	S. typhimurium	Titer	1.4 x 10 <sup>7</sup>	7	2 x 10 <sup>5</sup>	5 x 10 <sup>6</sup>
Hos		EOP	1	7	7	1
	DF1	Titer	2 x 10 <sup>7</sup>	3 x 10 <sup>6</sup>	5 x 10 <sup>5</sup>	6.5 x 10 <sup>6</sup>
1		Phage	~	Ffm	6SR	BR60

\* EOP = titer/ml S. typhimurium titer/ml DF1

Table 10. Titers and efficiency of plating of rough-specific enteric phages grown on  $\frac{S}{2}$ . Lyphimurium at  $22^{0}$ C and  $28^{0}$ C

		H.	Host 22°C				Host 28°C	
		urım	DF1		o. cypnimi	TI TO	1	DF1
Phage	Titer	EOP	Titer	EOP	Titer	Z0P	Titer	EOP
			•					
$Br_2$	1.4 X 10 <sup>7</sup>	Т	3 x 10	2.1	7.2 x 10 <sup>7</sup>	1	1 x 10	1 x 10 <sup>6</sup> 1.3 x 10 <sup>-2</sup>
Ffm	4.3 X 10 <sup>7</sup>	1	1.5 X 10 <sup>5</sup>	3.5 X 10 <sup>-3</sup>	5.5 x 10 <sup>7</sup>	1	1 x 10 <sup>5</sup>	1.8 x 10 <sup>-3</sup>
6SR	2 x 10 <sup>6</sup>	1	1 x 10 <sup>5</sup>	0.05	9 x 10 <sup>5</sup>	1	3 x 10 <sup>4</sup>	3,3 x 10 <sup>-2</sup>
BR60	1.5 x 10 <sup>6</sup>	1	1.5 x 10 <sup>5</sup>	0.1	1 x 10 <sup>6</sup>	1	1.5 X 10 <sup>3</sup>	1.5 x 10 <sup>-3</sup>

\* EOP = titer/ml DF1 titer/ml S. typhimurium

Table 11. Comparison of experimental and USP killed plague vaccines in mice for efficacy.

Number Y. pestis in challenge	Vaccine	Mg. nitrogen per ml	Vaccine dilution for 50% protective dose (PD <sub>50</sub> ) in mice
1,700 (214 mouse LD <sub>50</sub> )	USP Lot K9703	0.36	1:175
(214 100 105 105 50 7	ATCC 11953, 29 Aug 75 (0.05% formalin)	0.34	1:340
2,660	USP Lot M3793	0.39	1:22
(985 mouse LD <sub>50</sub> )	ATCC 11953, 29 Aug 75 (0.05% formalin)	0.39	1:210
	EV76S, 11 July 75 (0.05% formalin)	0.39	1:180
30,100	USP Lot M5826	0.40	1:5
(10,030 mouse LD <sub>50</sub> )	ATCC 11953, 29 Aug 75 (0.05% formalin)	0.40	1:63
	EV76S, 11 July 75 (0.05% formalin)	0.40	1:58
	ATCC 11953, 11 Feb 77 (0.05% formalin	0.40	1:49
	ATCC 11953, 11 Feb 77 (0.65% formalin)	0.40	1:23

Note: plague strain ATCC 11953 is strain Al122.

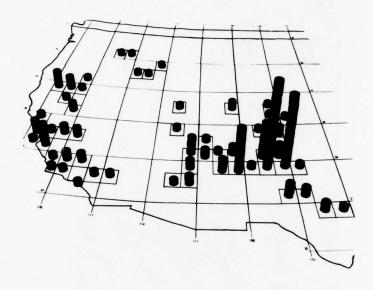


Figure 1. Geographical distribution of plague of probable sylvatic origin in the United States: 1920-1976. Grid coordinates based on "Major Army, Navy and Air Force Installations in the U.S.", Map 40-DMATC Series 8205, 2-76.



Figure 2. Rattus norvegicus with chronic plague infection. Animal has survived 24 months post infection. At autopsy, lesions contained virulent Y. pestis.

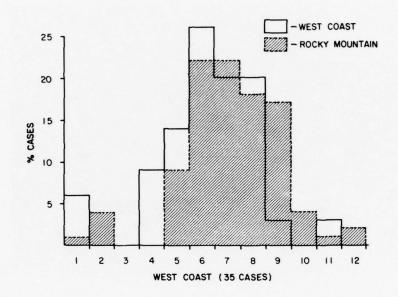


Figure 3. Seasonal distribution of plague cases of probable sylvatic origin in the United States: 1920-1976.

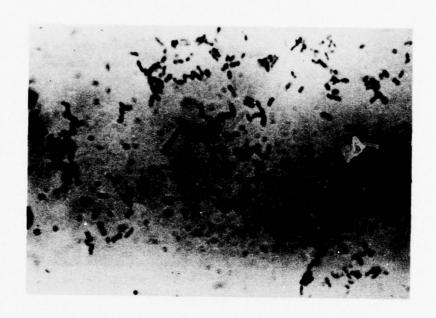


Figure 4. Peripheral blood of moribund plague patient: Wayson's stain.



Figure 5. Plague purpura in patient surviving infectious phase.



Electrophoretic patterns produced by various  $\underline{\mathrm{Y}}$ , pestis strains isolated in Bojolali, Java. Strain CO3311, the second strain from the right, lacks the heavy third band present in the other 7 strains. Figure 6.

Project 3M161102BS01 BASIC RESEARCH ON MILITARY DISEASES

Work Unit 133 Ecology of Plague

### Literature Cited.

### References:

- Anonymous Public Health Reports, Volumes 35-65, in part, 1920-1950.
- 2. Anonymous Morbidity and Mortality Weekly Reports, in part, Volumes 1-26, 1951-1977.
- 3. Anonymous. Historical statistics of the United States, Colonial times to 1970. Government Printing Office, Washington, D.C., 1975.
- 4. Butler, T. A clinical study of bubonic plague. Amer. J. Med. 53: 268-276, 1972.
- 5. Butler, T., and Hudson, B.W. The serological response to  $\underline{Y}$ . pestis infection. Bull. Wld. Hlth. Orgn.  $\underline{55}$ : in press, 1977.
- 6. Chen, T.H., and Meyer, K.F. Susceptibility of the langur monkey (Semnopithecus) to experimental plague: pathology and immunity. J. Infect. Dis. 115: 456-464, 1965.
- 7. Crumpton, M.J. and Davies, D.A.L. An antigenic analysis of Pasteurella pestis by diffusion of antigens and antibodies in agar. Proc. Roy. Soc. (London) B, 145: 109-134, 1956.
- 8. Davies, D.A.L. A specific polysaccharide of <u>Pasteurella pestis</u>. Biochem. J. (London) 63: 105-116, 1956.
- 9. Eisler, D.M., Kubik, G., and Preston, H. Colonial morphology and virulence of Pasteurella pestis. J. Bacteriol. 76: 41-47, 1958.
- 10. Eisler, D.M., Kubik, G., and Preston, H. Dissociation in Pasteurella pestis: Immunological comparisons of smooth and nonsmooth variants. J. Bacteriol. 76: 589-596, 1958.
- 11. Eisler, D.M., Kubik, G., and Preston, H. Dissociation in Pasteurella pestis: Interrelations of smooth and nonsmooth variants. J. Bacteriol. 76: 597-606, 1958.
- 12. Hudson, B.W., Quan, T.J., and Bailey, R.E. Electrophoretic studies of the geographic distribution of  $\underline{Y}$ . pestis protein variants. Intern. J. Syst. Bact.  $\underline{26}$ : 1-16, 1976.

- 13. Legters, L.J., Cottingham, A.J., Jr., and Hunter, D.L. Clinical and epidemiological notes on a defined outbreak of plague in Vietnam. Amer. J. Trop. Med. Hyg. 19: 639-652, 1970.
- 14. Meyer, K.F. The clinical and immunological responses of man to  $\underline{P}$ . pestis vaccine. Yugoslav. Acad. Sci. 1-82, 1971.
- 15. Meyer, K.F., Connor, C.L., Smyth, F.S., and Eddie, B. Chronic relapsing latent meningeal plague. Arch. Int. Med. <u>59</u>: 967-980, 1937.
- 16. Mullholand, B. Purpura and dry gangrene in plague. Proceedings of the 8th International Congress Trop. Med. Malaria, page 545, 1968.
- 17. Poland, J.D. Human plague from exposure to a naturally infected carnivore. International N.W. conference on diseases in nature communicable to man. Proceedings of the 27th Annual Meeting, Banff, Canada, 28-30 August, 1973.
- 18. Sites, V.R., Poland, J.D., and Hudson, B.W. Bubonic plague misdiagnosed as tularemia. J.A.M.A. 222: 1642-1643, 1972.
- 19. Von Reyn, C.F., Barnes, A.M., Weber, N.S., and Hodgin, U.G. Bubonic plague from exposure to a rabbit: a documented case, and a review of rabbit associated plague cases in the U.S. Amer. J. Epidem. 104: 81-87, 1976.
- 20. Wilkerson, R.G., Gemski, P. and Stocker, A.D. Non-smooth mutants of Salmonella typhimurium: differentiation by phage sensitivity and genetic mapping. J. Gen. Microbiol. 70: 527-554, 1972.
- 21. Williams, J.E., Harrison, D.N., and Cavanaugh, D.C. Cryptic infection of rats with nonencapsulated variants of  $\underline{Y}$ . pestis. Trans. Roy. Soc. Trop. Med. Hyg. 69: 171-172, 1975.
- 22. Williams, J.E., Harrison, D.N., Quan, T.J., Mullins, J.F., Barnes, A.M., and Cavanaugh, D.C. Atypical plague bacilli isolated from rodents, fleas and man. Am. J. Pub. Hlth., in press, 1977.
- 23. WHO Expert Committee on Plague. 4th Report, 1970. World Hlth. Org. Techn. Rep. Ser. No. 447, 1970.

### Publications:

- 1. Harrison, D.N., Dorsey, C.H., and Finley, H.F. Studies on a macronuclear endosymbiont of <u>Spirostomum ambiguum</u> I. Isolation of the microorganism from the macronucleus. Trans. Amer. Micros. Soc. <u>95</u>: 560-564, 1976.
- 2. Harrison, D.N., Dorsey, C.H., and Brown, C.A. Studies on a macronuclear endosymbiont of <u>Spirostomum ambiguum</u> II. Ultrastructural comparison of the in situ and the cultivated endosymbiont. Trans. Amer. Micros. Soc. <u>95</u>: 565-568, 1976.
- 3. Van Peenen, P.F.D., Joseph, S.W., Cavanaugh, D.C., Williams, J.E., Luyster, L.F., and Sulianti-Saroso, J. Absence of plague in certain mammals from Java and Kalimantan (Borneo). S.E. Asia J. Trop. Med. Pub. Hlth. 7: 411-414, 1976.
- 4. Williams, J.E., Imlarp, S., Top, F.H., Cavanaugh, D.C., and Russell, P.K. Kaeng Koi virus from naturally infected bedbugs (Cimicidae) and immature free-tailed bats. Bull. WHO <u>53</u>: 365-369, 1976.
- 5. Williams, J.E., Atas, M., and Cavanaugh, D.C. A comparison of serological tests for detecting antibody to plague. Bull. WHO  $\underline{54}$ : 232-233, 1976.
- 6. Williams, J.E., Eisenberg, G.H.G., Jr., and Cavanaugh, D.C. Decline of maternal antibodies to plague in Norway rats. J. Hyg., Camb. 78: 27-31, 1977.

#### Presentations:

- 1. Cavanaugh, D.C., and Williams, J.E. Plague: Some ecological interrelationships. Presented at the International Conference on Fleas. Ashton Wold, Peterborough, England, 21-25 June, 1977.
- 2. Cavanaugh, D.C. Plague. Presented at the Tropical Medicine Course, WRAIR, 18 Jul-26 Aug, 1977.

RESEARCH	AND TECHNOLOG	WORK UNIT S	UMMARY		A 6449	77 10			CONTROL SYMBO R&E(AR)636
76 10 01	D. Change	s. summary scty*	6. WORK SECURITY		ADING <sup>8</sup> Da	NL	OL SPECIF CONTRACT	OR ACCESS	A WORK UNIT
D. NO./CODES:*	61102A	3M161102BS			OO NUMBER	134	WORK U	NIT NUMBER	•
CONTRIBUTING	CARDS 114F								
	ological Mecha		licrobial I	nfect	ions				
SCIENTIFIC AND TE	chnological areas*								
3. START DATE	obiology oo.	14. ESTIMATED COM	PLETION DATE		HIG AGENCY		16. PERFO	RMANCE MET	HOD
62 08		CONT		DA		1		-House	
A DATES/EFFECTIVE:	NA	EXPIRATION:		10. RESC	PRECEDING	TE & PROF	ESSIONAL MAN	RS & FUI	DS (In thousands)
NUMBER:*				FISCAL	77	3	.0		279
C TYPE:		d AMOUNT:		YEAR	78	5	0		211
9. RESPONSIBLE DOD	ORGANIZATION	I.COM. AMT.		20. PERF	ORMING ORGAN		1		1
FOREIGN INTERPRETATIONS  FOREIGN INTERPRETATION  EXEMPTOR OF (U) Complem  TECHNICAL OBJECT  23 (U) The the natural of military the develop research as	on Rapmund, (	considere considere (U) Radio PROGRESS (Pumlet 1 this work al inducti This incl dologies f prostic eva	Immunity; immunoassa, unit is to on of immu udes the s for the stu- luations.	NAME:  TELEP SOCIAL ASSOCIA HAME: HAME: (U) A y; (U millied b, to elu nity tudy dy of	Carter HONE: 202- SECURITY ACC TE INVESTIGAT  INTIBODIC  Serod  Cidate i to a vai of infec the imm	L. Digg -576-356 count number one es; (U) iagnosis the mech riety of ctions	Infection  Infection	ous Di	seases; ive in fections ms and ns for
parameters in vitro ex common to a 25 (U) 76 induce perm proteins. activate th	of disease are periments. As variety of control of the control of	nd of the invariety of the different of	mmune respond diseases are hown, using eraction was membrane pathway.	onse are e als g mod ith g chole For t	to disea attacked o studie el membranglios sterol l echnica	ase both d. Immu ed. ranes, d ide in d nave bee	that cho the abse	vivo and all phenology of the phenology	nd nomena n can other able to eed

Project 3M161102BS01 BASIC RESEARCH ON MILITARY DISEASES Work Unit 134 Immunological Mechanisms in Microbial Infections

Investigators: LTC Carl Alving, MC, M.D., Roberta Richards, Ph.D.

Assistants: SP5 Katharine Urban, M.S., B.A.

A. Light-Induced Leakage of Spin Label Marker from Liposomes in the Presence of Phototoxic Phenothiazines

Liposomes prepared from dipalamitoyl lecithin, cholesterol and dicetyl phosphate and containing a trapped spin label marker were exposed to long wavelength UV light in the presence of series of phenothiazine tranquilizers. EPR spectroscopy was used to detect spin label marker released from liposomes taking advantage of the disappearance of line broadening from electron spin exchange which occurred on spin label release. The minimum effective phototoxic dose in mice of the phenothiazines was also determined. Kinetic studies of light-induced spin label release from phenothiazine-sensitized liposomes showed that membrane damage was rapidly induced and that the damaging species were shortlived. The damage process was oxygen dependent and could be temporarily prevented by cysteamine or α-tocopherol added immediately before irradiation. Only those phenothiazines which mediated light-dependent liposomal membrane damage had phototoxic activity in mice and the degree of photosensitization was parallel in the two systems. In both photosensitization phenomena, the nature of the substituent at the phenothiazine 2-position was more important than the phenothiazine side chain.

B Effect of the A and B Protomers of Choleragen on Release of Trapped Glucose from Liposomes Containing or Lacking Ganglioside G<sub>M1</sub>

Liposomes containing trapped glucose were used to examine the interaction of the A and B protomers of choleragen with ganglioside  $G_{\tt Ml}$  and lipid model membranes. The B protomer (choleragenoid) was as effective as choleragen in causing release of trapped glucose from liposomes containing  $G_{\tt Ml}$ ; the A protomer did not release glucose from  $G_{\tt Ml}$  liposomes. Neither choleragen nor the A or B protomers caused release of trapped glucose from glycolipid-free liposomes. Anti-choleragen and complement, however, caused release of trapped glucose from ganglioside-free liposomes previously incubated with the A protomer but not from those incubated with the B protomer or choleragen. These results suggest that the A protomer, but not intact choleragen or B protomer, bound to ganglioside-free liposomes. Presumably , the A protomer must be freed of the constraints present in the intact choleragen in order to interact with the liposomal model membrane system.

C. Choleragen-mediated Release of Trapped Glucose from Liposomes Containing Ganglioside  $\mathbf{G}_{\texttt{M1}}$ 

125I-Labeled choleragen was bound to liposomes containing galactosyl-N-acetylgalactosaminyl-(N-acetylneuraminyl)-galactosylglucosylceramide (G M1 ), but not in large amounts to ganglioside-free liposomes nor to those containing N-acetylneuraminylgalactosyglucosylceramide (G<sub>M</sub>) N-acetylgalactosaminyl Nacetylneuraminyl) galactosylglucosylceramide (GM2) or N-aceylneuraminylgalactosyl-N-acetylgalactosaminyl-l Nacetylneuraminyl) galactosylglucosylceramide (GDla). Choleragen released trapped glucose only from  $G_{\mbox{\scriptsize Ml}}$ -liposomes. This choleragen-induced glucose released from  $G_{M1}$  -liposomes was relatively rapid for the first few minutes, then continued more slowly. The amount of glucose released from liposomes in 30 min was dependent on both the  $G_{M1}$ content and choleragen concentration. Prior incubation of GM1-liposomes with anti-G<sub>Ml</sub> antiserum prevented the choleragendependent release of trapped glücose. After incubation of G<sub>M1</sub>-liposomes with choleragen, addition of anti-choleragen antibodies and complement led to more extensive glucose release. Under these latter conditions a much smaller glucose release was observed also from liposomes containing G MJor N-acetylneuraminylgalactosyl-N-acetylgalactosaminyl-(N-acetylineuraminyl)-galactosylglucosylceramide in the absence of choleragen. These releases were attributed to naturally-occurring antiganglioside antibodies in the antiserum and complement. Ganglioside-free liposomes did not release glucose in response to anticholeragen and complement. It appears that choleragen in the absence of other proteins binds specifically to liposomes containing  $G_{M1}$  and can induce permaeability chang-

D. Uptake of Ganglioside G<sub>M1</sub> into Preformed Liposomes, and Complement-Dependent Glucose Release in the Presence of Anti-G<sub>M1</sub> of anti-choleragen.

The monosialoganglioside  $G_{\mbox{\scriptsize Ml}}$  , which is the cell surface receptor

for choleragen (cholera toxin), was taken up by preformed liposomes (containing lecithin, cholesterol, and dicetyl phosphate). Excess  $G_{\mbox{\scriptsize Ml}}$ , was removed by centrifuging the liposomes. Uptake was monitored by incorporation of ( $^3\mbox{\scriptsize H})\mbox{-}G_{\mbox{\scriptsize Ml}}$  binding of ( $^{125}\mbox{\scriptsize I})\mbox{-}choleragen, or release of trapped glucose by the addition of anti-GM antibodies plus complement or choleragen, anti-choleragen, and complement. G uptake was greater at 45 C than at 25 C and at 18 hr than at 15 min. Uptake was similar with liposomes containing either dimyristoyl or dipalmitoyl-lecithin. Of the immunochemical methods, the choleragen-anti-choleragen assay was found to be much more sensitive than the Anti- <math display="inline">G_{\mbox{\scriptsize Ml}}$  assay for de-

tecting small amounts of  $G_{M1}$  in liposomes. Presumably, this was because choleragen, unlike  $G_{M1}$ , is a multivalent antigen and may have bound more antibodies, resulting in greater complement fixation and glucose release.

We conclude that  $G_{M1}$  can be absorbed in large amounts by preformed liposomes. Incorporation of the ganglioside can be detected by complement-dependent glucose release in the presence of choleragen and anticholeragen or, with less sensitivity, in the presence of anti- $G_{M1}$  antibodies.

E. Interactions of C-Reactive Protein and Complement with Liposomes.

C-reactive protein (CRP) has phosphorylcholine binding specificity. Incubation of CRP with liposomes containing dimyristoylphosphatidyl choline, cholesterol, stearylamine (to provide a strong positive charge), and galactosylceramide, resulted in complement-dependent release of trapped glucose. Glucose release was inhibited by soluble phosphorylcholine. Liposomes either having a negative charge, or lacking galactosylceramide, did not release glucose, even though hemolytic complement was fixed. Damage to liposomes did not occur in the presence of EGTA, or in the presence of C2- or C7- deficient human serum, but did occur when these deficient sera were combined.

CRP caused consumption of hemolytic complement in the presence of liposomes containing egg phosphatidylcholine, and this occurred whether the liposomes had a positive, neutral or negative charge, or whether they contained or lacked a galactosylceramide. These interactions with CRP resulted in consumption of each of the first four complement components.

We conclude that liposomes may be useful as models for studying CRP complement interactions with membranes. CRP can bind to liposomes, causing classical pathway complement activation which may, or may not, result in membrane damage; these effects are influenced by the membrane lipid composition.

## F. Interactions of Plant Lectins with Glycolipids in Liposomes

A panel of five plant lectins with different binding specificities was used to determine if plant lectins could bind specifically to membrane associated glycolipids. Ricinis communis and wheat germ agglutinins both bound specifically to mixed brain gangliosides and globoside I from human erythrocytes. Wheat germ agglutinin also bound to ganglioside  $G_{\mbox{\scriptsize Ml}}$  and human erythrocyte ceramide trihexoside, but not to ceramide dihexoside, mono-, or digalactosyl diglycerides. Concanavalin A bound to liposomes with or without glycolipid substiuents, and this binding was partially inhibited by  $\alpha\text{-methyl}$  mannoside. This study indicates that lectins can specifically recognize and bind to certain glycolipids in membranes.

# G. Immune Reactivities of Antibodies Against Glycolipids

A new, simple technique is described for purifying specific antiglycolipid (galacatocerebroside) antibodies from antiserum. The antibodies first were absorbed to galactocerebroside-containing liposomes and then were recovered, after washing the liposomes, by elution with lM NaI, or by extraction with saline-chloroform mixture. Depending on the antiserum used, this procedures resulted in approximately a 175fold to 3800-fold purification of antibodies from whole serum when calculated on an activity/protein basis. The antibodies were tested for activity by means of complement-dependent glucose release from separate galactocerebroside containing indicator liposomes. It was found that the hydrophobic portions both of the galactocerebroside and the surrounding phospholipids had an influence on antibody binding. The relative anti-galactocerebroside activity, as determined by liposomal glucose release, was directly related to the galactocerebroside fatty acyl This suggested that the presence of a longer fatty acyl chain in the glycolipid molecule made the carbohydrate more accessible to binding with specific antibody. Furthermore, the degree of adsorption of antibody activity from the antiserum was progressively decreased when the hydrophobic region of the phospholipid membrane was increased due to the presence of phospholipids containing progressively longer fatty acids. These results were consistent with the hypothesis that under certain conditions the immunologically-active group on galactocerebroside may be partially "buried" in the phospholipid bilayer and may be less available for antibody binding.

With certain antisera, the antibodies eluted from liposomes having longer phospholipid fatty acids (distearoyllecithin) had higher avidity for galactocerebroside than did those from liposomes having shorter phospholipid fatty acids (dimyristoyllecithin). In contrast, a long fatty acyl chain of galactocerebroside (lignoceroyl galactocerebroside) resulted in eluted antibodies having a lower avidity than when a shorter glycolipid (stearoyl or palmitoyl galactocerebroside) was used. It was thought that that these results were caused by preferential binding of high affinity antibodies under conditions in which binding was partially hindered due to steric conditions.

Specific antibodies against ceramide-monohexoside (CMH), ceramide dihexoside (CDH) and ceramide trihexoside (CTH) were purfied from antiserum or normal (unimmunized) rabbit serum by using liposomes as immunoadsorbents. Purified antibodies were compared with whole antiserum, or immunologically reactive normal rabbit serum, for specific activity against the immunizing antigen, and cross-reactivity with the other haptens. High levels of reactivity and reciprocal cross-reactivity were observed both with antisera and purified antibodies against CMH and CDH. In contrast, although anti-CMH and anti-CDH sera both apparently "cross-reacted" with CTH, the purified antibodies in each case did not have any detectable cross-reactivity with CTH.

Conversely, anti-CTH antiserum apparently "cross-reacted" with CDH, whereas purified antibodies did not. It was concluded that rabbit antiserum sometimes may contain naturally-occurring antibodies against glycosphingolipid, and these may be eliminated by using purified non-cross-reacting antibodies. Naturally-occurring antibodies against CDH and CTH, but not against CMH were found in 10 normal rabbit sera. Purified naturally occurring anti-CDH antibodies obtained from one of these rabbits did not cross-react with CTH, but purified anti-CTH from the same rabbit did cross-react with CDH.

Pools of "high activity" or "low activity" anti-CMH antibodies were obtained from a single rabbit by using antisera taken at different times in the immunization schedule. These antibodies were tested for activity against CMH and CDH (both having a terminal B-galactose), and CTH and digalactosyl diglyceride. Based on this it was concluded that anomeric configuration alone did not necessarily determine specificity of purified antibodies. In contrast, the low activity antibodies, in quantities which gave the same high degree of reactivity with the immunizing antigen observed with high activity antibodies, showed only very low levels of cross reactivity with either CDH or digalactosyl diglyceride. It was concluded further, therefore, that cross-reactivity may depend to some extent on relative antibody activity (which may be related to antibody affinity) against the immunizing antigen.

H. Cholesterol-dependent Human Complement Activation Resulting in Damage to Liposomal Model Membranes

Human (but not guinea pig) complement was activated spontaneously by liposomes containing a high concentration (71 mol %) of cholesterol. This occurred in the absence of any recognizable antigen or antibody, and did not occur at a low concentration (43 mol %) of cholesterol. Activation of complement resulted in membrane damage and release of trapped liposomal glucose. The complement activity was inhibited by preheating (56 C, 30 min), 10 mM Mg EDTA or EGTA, and by prior absorption with insoluble immune complexes. Almost all human sera had some reactivity, but it ranged from very low levels ( 7% liposomal glucose release) to very high levels ( 50% glucose release). Complement activation appeared to be mediated by a serum factor which could be removed by adsorption and which was partially heat labile. The factor was transferred by adding heated high reacting human serum to unheated low reacting human serum, or to guinea pig serum. The serum factor although quantitatively diminished in potency due to heat lability, caused equal activation of each of these two latter complement sources in the presence of high cholesterol liposomes. It did not cause activation of C4-deficient guinea pig complement. These data suggest that the classical complement pathway was activated. The liposomal membrane composition had an influence on this phenomenon. Activities of about half of the human sera were enhanced when galactosylceramide, or ceramide alone was present in the liposomes. Activity was enhanced by longer fatty acyl chain lengths of lecithin when dimyristoyl-dipalmitoyl-, or distearoyllecithin was employed in the liposomes. Liposomes containing sphingomyelin as the only phospholipid were not sensitive to cholesterol-dependent complement-mediated damage. It was concluded that human complement was activated in the presence of high concentrations of membrane cholesterol and that this was caused by an uncharacterized serum factor and was influenced by the lipid composition of the membrane.

Project 3M161102BS01 BASIC RESEARCH ON MILITARY DISEASES

Work Unit 134 Immunological Mechanisms in Microbial Infections

## Literature Cited.

## References:

# Publications:

- l. Copeland, E.S., Alving, C.A., and Grenan, M.M.: Light-induced leakage of spin label marker from liposomes in the presence of phototoxic phenothiazines. Photochemistry and Photobiology, 24: 41. 1976.
- 2. Moss, J., Richards, R.L, Alving, C.R., and Fishman, P.H.: Effect of A and B protomers of choleragen on release of trapped glucose from liposomes containing or lacking ganglioside G. The Journal of Biological Chemistry, 252: 797, 1977.
- 3. Moss, J., Fishman, P.H., Richards, R.L., Alving, C.R., Vaughan M., and Brady, R.O: Choleragen-mediated release of trapped glucose from liposomes containing ganglioside G. Proc National Academy Science. 73: 3480, 1976.
- 4. Boldt, D.H., Speckart, S.F., Richards, R.L. and Alving, C.A.: Interactions of plant lectins with glycolipids in liposomes. Biochemical and Biophysical Research Communications. 74: No. 1, 1977.
- 5. Alving, C.R., Richards, R.L.: Immune reactivities of antibodies against glycolipids-I. Immunochemistry. 14: 373, 1977.
- 6. Alving, C.R., Richards, R.L.: Immune reactivities of antibodies against glycolipids-II. Immunochemistry. 14: 383, 1977.
- 7. Alving, C.R., Richards, R.L.: Cholesterol-dependent human complement activation resulting in damage to liposomal model membranes. The Journal of Immunology. 118 342, 1977.
- 8. Richards, R.L., Fishman, P.H., Moss, J., and Alving, C. R: Uptake of ganglioside G into preformed liposomes, and complement-dependent glucose release in the presence of anti G or choleragenanti-choleragen. Federation of American Societies for Experimental Biology, 3010, 1977.
- 9. Alving, C.R., Richards, R.L., and Gewurz, H.: Interactions of C-reactive protein and complement with liposomes. Federations of American Societies for Experimental Biology, 5271, 1977.

DESTADON	AND TECHNOLOGY	, money 11117 6		1. AGEN	CY ACCESSIO	10 2	DATE OF SU	MMARY	REPORT	CONTROL SYMBOL	
RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY					DA OB 6513		77 10 01		DD-DR&E(AR)636		
1. DATE PREV SUMPRY	4. KIND OF SUMMARY	S. SUMMARY SCTY	S. WORK SECURITY				P'N INSTR'N	SE SPECIFIC	BACCESS	. LEVEL OF SUM	
76 10 01	D. Change	U	U		NA L		NL		□ №0	A. WORK UNIT	
10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER		R	WORK UNIT NUMBER				
& PRIMARY	61102A	3M161102B	-00			135					
b. CONTRIBUTING	CADDC 3345					_					
c. XXXXXXXXXXXXX	Security Classification Code						-				
(U) Mechanisms of Transmission of Hepatitis Viruses 12. SCIENTIFIC AND TECHNOLOGICAL AREAS											
002600 Biology 010100 Microbiology 003500 Clinical Medicine 13. START DATE [14. ESTIMATED COMPLETION DATE ] IS FUNDING AGENCY [14. PERFORMANCE METHOD											
				IS FUNDING AGENCY IS PERFORMANCE METHOD							
	72 07 CONT			DA			C. In-House				
17. CONTRACT/GRANT				10. RESOURCES ESTIMATE			& PROFESSIONAL MAN YRS b. FUNDS (In thousands)				
A DATES/EFFECTIVE:	NA	EXPIRATION:			PRECEDING		2.5			133	
b. NUMBER:*		4 AMOUNT:		FISCAL CURRENT			1		155		
e. KIND OF AWARD:				1500	78		2.5	5		68	
19. RESPONSIBLE DOD	DRGANIZATION	f. CUM. AMT.		20. PER	ORMING ORG	ANIZA					
"AME: Walter Reed Army Institute of Research Div of CD&I								f Research			
ADDRESS: Washington, D.C. 20012					ADDRESS:* Washington, D.C. 20012						
					AL INVESTIGA	TOR	Furnish SEAN	II U.S. Academi	c [netitution	,	
RESPONSIBLE INDIVIDUAL					MAME: BANCROFT, LTC William H.						
NAME: RAPMUND, COL Garrison					TELEPHONE: (202) 576-3757						
TELEPHONE: (202) 576-3551					SOCIAL SECURITY ACCOUNT NUMBER:						
21. GENERAL USE					ASSOCIATE INVESTIGATORS						
Foreign intelligence not considered					MAME: TOP, COL Franklin H., Jr.						
22 KEYWORDS (Procedo	NAME: IFMON, MAJ Stanley M.										
(U) Viruses: (U) Hepatitis: (U) Antigen: (U) Immunology  23. TECHNICAL OBJECTIVE.* 24. APPROACH, 25. PROGRESS (Pumish Individual paragraphs Identified by number. Proceeds text of sect with Security Classification Code.)											
23 (U) To define the epidemiology of hepatitis in military populations in order to											
establish methods for reducing disability from hepatitis. Emphasis is on developing											
and applying sensitive and specific methods for detection of hepatitis viruses - anti-											
gens and antibodies to determine host factors important in resistance to disease and											
infection in military personnel.  24 (U) New methods for identification and antigenic analysis of hepatitis viruses are											
under development. The immune response of patients infected with hepatitis viruses											
is studied to define sensitive parameters of infection and to define factors critical											
in immunity. The epidemiology of hepatitis B in military populations is defined.											
25 (U) 76 10 - 77 09 A search was made for a source of antigen and antibody to use for											
the development of an assay for hepatitis A virus. An epidemic of hepatitis A at Ft.											
Richardson, Alaska was investigated in collaboration with the Division of Preventive											
Medicine, W	RAIR. A chil	d care cent	ter on post	was	implic	ate	d as th	ne princ	cipal	source	
of infection	n. Four adul	ts were pla	smapherese	d to	obtain	1a	rge vo	lumes o	f hepa	titis A	
antibody.	Human fecal s	pecimens we	ere collect	ed fi	rom cas	es	of rece				
tomatic fam	ily contacts	of cases in	order to	obta.	in anti	gen	. Feca			re tested	
for antigen using a solid phase radioimmune assay and the human immune plasma. Only											
two samples from one individual were reactive as antigen. Another source of hepatitis											
A antigen is being sought. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 76 - 30 Sep 77.											
nesearch Annual Progress Report, 1 Jul 70 - 30 Sep 77.											
2101											
			56	4							
*Available to contractors upon originator's approval.											
DD FORM 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 68 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.											

Project 3M161102BS01 BASIC RESEARCH ON MILITARY DISEASES
Work Unit 135 Mechanisms of transmission of hepatitis viruses
Investigators:

Principal: LTC W.H. Bancroft, MC; CPT R.G. Allen, MSC

Associate: LTC M.W. Benenson, MC; SSG D.A. Leach

SP4 J.R. Putnak; H.G. Cannon

# Description

To define the epidemiology of hepatitis in military populations in order to establish methods for reducing disability from hepatitis. Emphasis is on developing and applying sensitive and specific methods for detection of hepatitis viruses/antigens and antibody to determine host factors important in resistance to disease and infection.

# Progress

- I. Hepatitis A Virus
  - A. Acquisition of Materials for Studying Hepatitis A Virus

# 1. Background

Between August 1976 and February 1977, an epidemic of Hepatitis A involving at least III people occurred at Ft. Richardson, Alaska. The epidemic was investigated by an EPICON team from the WRAIR led by LTC Michael Benenson, Division of Preventive Medicine. Epidemiological investigation revealed that most cases of hepatitis occurred in married couples who had children attending the Child Care Center (CCC) on post. It was concluded that hepatitis A virus was probably introduced to the CCC, perhaps by an adult, and transmitted through close contact to the children, who subsequently infected their parents. A complete review of the epidemiological investigation may be found elsewhere in this annual report under the Division of Preventive Medicine.

The epidemic was considered to present an unusual opportunity for the Department of Virus Diseases to obtain human fecal and blood specimens and antibody (anti-HAV). Specimens were collected over a 10-day period in December 1976 for these purposes.

### 2. Methods

a. Collection of Specimens

Other investigators have found fecal shedding of Hepatitis A virus to occur prior to the onset of symptomatic disease. IgM antibody detectable by radioimmunoassay has been found in acute blood samples while IgG antibody detected by immune adherence hemagglutination tests usually appears about 10 days after the onset of illness. Based upon this information, specimens were collected in the following manner.

Daily fecal specimens were solicited from all family contacts of acute cases who had an onset of illness within the preceding week. Fecal specimens were collected in unsterile cardboard cartons, labelled with name and date, placed in a plastic bag, and refrigerated outside in a snowdrift until picked up for storage. Specimens were stored in dry ice within 36 hours of collection for transportation to the WRAIR. Fecal specimens were obtained from three recent cases of hepatitis (12 specimens) and 15 family contacts of recent cases (33 specimens).

Four adults who had fully recovered from hepatitis were plasmapheresed to obtain large quantities of immune plasma to use for antigen detection. Three of the donors had been found to have anti-HAV in serum samples collected in November by LTC Benenson and tested by Dr. Robert Purcell, NIAID. Plasmapheresis was performed in the blood bank of Elmendorf AFB Hospital. Two units of plasma (approximately 300 ml each) were obtained from each of three people and one unit from the fourth person.

Paired sera were obtained from 10 cases of acute hepatitis confirmed by interview and biochemical tests of liver function. Initial serum samples were collected 4 to 19 days after the onset of disease. Complete records of blood chemistries were also obtained.

In addition, serum specimens were obtained from 23 employees of the Ft. Richardson CCC, 26 employees of the Elmendorf AFB CCC and 12 employees of the Elmendorf preschool nursery.

# b. <u>Characteristics of Immune Plasmas</u>

The clinical histories of the four plasma donors can be summarized as follows: Patient AKOO3, Mr. C.S., was a 23-year-old active duty Army enlisted man who developed symptoms of fever on 16 August followed by nausea, vomiting, abdominal pain, malaise and anorexía. By 25 August, he was jaundiced and had dark urine. Laboratory values on 23 August were: SGOT 1060, LDH 466, alkaline phosphatase 52, and bilirubin 3.5/4.8. On August, the SGOT was 81, LDH 93, alkaline phosphatase 70 and bilirubin 1.3/2.0.

Patient ASO05, Mrs. S.L., was a 27-year-old Army dependent wife and employee of the Ft. Richardson CCC who developed symptoms of nausea, vomiting, fever, abdominal pain, diarrhea, and anorexia on 23 August 1976. Jaundice appeared on 2 September. Laboratory values for 30 August were: SGOT 865, LDH 135, bilirubin 3.2/6.5. On 24 September, the SGOT was 13 and the bilirubin was 0.5/1.3.

Patient AKO19, Mr. J.H., was a 26-year-old active duty Army officer who developed symptoms of fatigue, anorexia, nausea, vomiting, fever and diarrhea on 19 October 1976. He subsequently developed jaundice, dark urine and light-colored stools. Laboratory values for 27 October were: SGOT 158, LDH 102, alkaline phosphatase 116, SGPT 181, and bilirubin 4.3/10.1. Values for 3 November were: SGOT 60, alkaline phosphatase 106, SGPT 230 and bilirubin 0.9/3.2.

Patient AK059, J.B., was a 29-year-old dependent wife living on Elmendorf AFB and employed by the Ft. Richardson CCC. She had the onset of symptoms on 20 October with headache, nausea, vomiting, fatigue, abdominal pain, anorexia and diarrhea. Her laboratory values on 27 October were SGOT 101, LDH 152, alkaline phosphatase 140 and bilirubin 0.5/1.0. The serological results for the four plasma donors are listed in Table 1.

# c. Assay of Fecal Samples for HAV

Each fecal specimen was suspended in normal saline (20% w/v), mixed thoroughly and centrifuged at low speed. The supernate was tested for HAV by solid phase radioimmune assay (RIA), using the same method as is used for the detection of anti-HBc (annual report 1976). Whole immune plasma was used to coat the wells in plastic plates. The IgG portion of plasma AK005 was separated and radiolabelled with  $^{125}\text{I}$  to use as the outer layer of the RIA "sandwich."

# d. Detection of Anti-HAV

All tests for anti-HAV in sera were done by RIA inhibition by Dr. Blaine Hollinger, Baylor University.

### Results

### a. Detection of HAV

Forty-five fecal samples were screened for reactivity with the immune plasmas. Although several specimens reacted in some tests, only the stools from one person were repeatedly reactive. J.S.W., a 6-year-old son of a woman who developed acute hepatitis on 1 December, did not become symptomatic himself, but had a stool specimen on 12 December which reacted with the immune plasma. Dr. Hollinger tested all five stool specimens from the boy and found low level antigen activity in samples from 11 and 12 December. Additional work is needed to improve the method of purification of Hepatitis A antigen.

# b. Detection of Anti-HAV

Dr. Hollinger found anti-HAV in 8/8 people with symptomatic acute hepatitis. Among asymptomatic employees of the Ft. Richardson and Elmendorf Child Care Centers, the prevalence of antibody was 15/23

(65%) and 15/25 (60%), respectively. However, none of 8 employees of the Elmendorf preschool had high levels of anti-HAV. The results suggest that hepatitis A may have been transmitted to personnel at the Elmendorf CCC as well as at Ft. Richardson.

### II. Hepatitis B Virus

Work on Hepatitis B virus was confined to the detection of hepatitis B surface antigen HBsAg and antibody (anti-HBs) in selected groups of sera by commercial radioimmunoassay upon request. "e" antigen and antihepatitis B core tests were done as needed by standard procedures.

In a collection of sera obtained from members of Special Forces and the 82nd Airborne Division deployed from Ft. Bragg, N.C. to Alaska in January 1977, the prevalence of HBsAg and anti-HBs was 2/273 (0.7%) and 41/273 (15%), repectively. None of the troops developed symptoms of hepatitis during one month of follow up. Hepatitis A antibody tests were not done since all personnel had received immune serum globulin in advance.

Table 1. Hepatitis Test Results for Four Donors of Immune Plasma Ft. Richardson, Alaska, 1976

Study Number		Blood Sample			HBV	Ant	i-HAV*
Patient ——————	No.	Serum/Plasma	Date	HBsAg	Anti-HBs	IAHA	RIA % inhit
AK003 C.S.	EX 217	P	13 Dec	N**	N	16000	
AK005	EX 218	S	16 Nov	N	N	≽16000	98
S.L	EX 219	S	17 Nov				95
	EX 220	S	14 Dec		N	≥16000	90
		P	16 Dec			20000	
AK019	EX 228	S	14 Nov	N	N	≽1600	
J.H.	EX 229	P	10 Dec		N	4000	
AK059	EX 265	S	15 Nov	N	N	400	99
J.H.	EX 266	P	11 Dec		N	4000	98

<sup>\*</sup> Immune adherence hemagglutination (IAHA) was done by Dr. Robert Purcell, NIAID, NIH. RIA inhibition test was done by Dr. Blaine Hollinger, Baylor University.

<sup>\*\*</sup> N equals negative.

Project 3M161102BS01 BASIC RESEARCH ON MILITARY DISEASES
Work Unit 135 Mechanisms of transmission of hepatitis viruses

### Publications:

Bancroft, W.H., Snitbhan, R., Scott, R.M., Tingpalapong, M., Watson, W.T., Tanticharoenyos, P., Karwacki, J.J., and Srimarut, S. Transmission of hepatitis B virus to gibbons by exposure to human saliva containing hepatitis B surface antigen. J. Inf. Dis. 135: 79-85, 1977

Irwin, G.R., Allen, A., Segal, H., Willhight, M., Cannon, H., and Top, F.H., Jr. Persistence of antibody to hepatitis B surface antigen. J. Clin. Microbiol. 3: 465-468, 1976

Irwin, G.R. Passive immunization against exposure to hepatitis B virus in the military: potential and possibilities. Yale J. Biol. Med.  $\underline{49}$ : 251-257, 1976

Irwin, G.R., Allen, R., Segal, H., Allen, A., Putnak, J., Cannon, H. and Top, F.H., Jr. J. Infec. Dis. 136: 31-36, 1977

RESEARCH	AND TECHNOLOGY	WORK UNIT SI	JMMARY	DA O				&E(AR)636	
1. DATE PREV SUM'RY	4. KIND OF SUMMARY	S. SUMMARY SCTY	. WORK SECURITY	7. REGR			ON TRACTO	R ACCESS	LEVEL OF SUM
76 10 01	D. Change	U	U	NA		NL	K YES	□ MO	A WORK UNIT
10. NO./CODES:*	PROGRAM ELEMENT	PROJECT			REA NUMBER			IT NUMBER	
& PRIMARY	61102A	3M161102B	501	0	0		136	***************************************	
b. CONTRIBUTING	CARDS 11/15					-			
c. XXXXXXXXXXXX	CARDS 114F								
(U) Devel	opment of Bio	logical Pro	oducts						
010100 Mic		14. ESTIMATED COMP	LETION DATE	TIS FUNC	NIG AGENCY		14. PERFOR	MANCE METH	100
58 05		CONT		DA		4	c. 1	n-House	e
				10. RES	PRECEDING	E & PROFESSIO	MAL MAN Y	es & FUNC	OS (In thousands)
A DATES/EFFECTIVE:	NA	EXPIRATION:		FISCAL	77	4		1 .	382
C TYPE:		4 AMOUNT:		YEAR	CURRENT			+	302
& KIND OF AVARD:		f. CUM. AMT.		,	78	4			357
19. RESPONSIBLE DOD	DRGANIZATION			20. PERI	ORMING ORGANI				Ť
	Reed Army In	stitute of	Research	HAME:*	Walter Re	eed Army		ute of	Research
PRINCIPAL INVESTIGATOR (Pumleth SSAN II U.S. Academic Innitiation)  HAME: Rapmund, COL Garrison  TELEPHONE: 202-576-3551  21. GENERAL USE  ASSOCIATE INVESTIGATORS  HAME: Berman, S. L., Ph.D.  Foreign intelligence not considered  HAME: Altieri, P. L.  DA					DA				
(U) Bioass	ays; (U) Biolo	ogical Prod	lucts; (U)	Dengi	ue Virus	Vaccine;	(U) F	ebrile	Antigens
23. (U) The product of existing afford grea	his work unit ion of new ef biological p ter stability	is concern fective vac roducts to , and to mi	ned with th cines for increase e nimize log	e dev milit ffect istic	relopment ary use iveness require	t of manum , and with and reduce ements.	facturi h the r ce read	ing met modific ctivity	thods and cation ,, to
physical and of logistic		thods for p	rocessing.	1 mp	rovement	t in stab	ility a	and red	duction
of logistic requirements are achieved by application of modern freeze-drying and packaging techniques.  25. (U) 76 10 - 77 09 - Investigations on the development of new and improved biological products for military use have continued 1. Laboratory studies on meningococcal vaccines have resulted in improvement of methods for the production of a group B protein-polysaccharide complex and the production of purified polysaccharides from Group Y and strains 29E and 135 for use as immunogens. 2. Two experimental lots of inactivated plague vaccine have been prepared from attenuated strains for evaluation in animals. 3. Studies on the development of vaccines for dengue viruses, types 2 and 3, have continued. Work was also initiated on the development of a mouse potency assay for the evaluation of dengue vaccines. 4. An investigation of the stability of tableted diagnostic antigen preparations was carried out. 5. Studies on the addition of iron to substandard lots of mucin to enhance the virulence of challenge suspensions in mouse potency assays for bacterial vaccines have continued. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 76 - 30 Sept 77.									

Project 3M161102BS01 BASIC RESEARCH ON MILITARY DISEASES

Work Unit 136 Development of biological products

Investigators.

Principal: Joseph P. Lowenthal, ScD.

Associate: Sanford Berman, PhD.; Patricia L. Altieri, B.S.; Doria

R. Dubois, M.S.; Calvin J. Powell, M.S.; Melanie G. Butler, B.S.; SP5 Edward Cieslak, B.S.; Clayton R. DeSett, B.S.; Clayton Hartley, B.S.; Quesada Jackson, SP4 Patricia B. Matix, B.S.; Robert L. Timchak; Gary A.

Vincent, B.S.; Bernard Young.

### Description.

This work unit is concerned with the development of manufacturing methods for the production of new effective vaccines for military use, and with the modification of existing biological products to increase effectiveness and reduce reactivity, to afford greater stability, and to minimize logistic requirements.

# Progress.

# 1. Meningococcal Vaccines.

During the past year laboratory studies were continued on the development of methods for the preparation of a protein-polysaccharide complex antigen derived from the group B meningococcus for possible use in the immunization of man against this type of meningitis. Investigations were also continued on procedures for the production of purified polysaccharides from the Boshard strain (group Y) of Neisseria meningitidis for use as an immunogen. In addition, studies were initiated on N. meningitidis strains 29E-106 and 135-III for the purpose of obtaining appropriate polysaccharide immunogens.

a. The investigations on the production of a protein-polysaccharide antigen suitable for human use prepared from the B-11 strain of N. meningitidis (Annual Report, 1976) have led to the preparation of two vaccines, one produced by personnel of the Department of Biologics Research, WRAIR (Lot PA-3) and the other by personnel of the Department of Bacterial Diseases, WRAIR (Lot WZ-2).

For both vaccines, cultures of the B-11 strain were grown in a modification of Franz's medium in which the casamino acid component is increased 3-fold and the dextrose concentration reduced to one fifth that of the original medium (Annual Report, 1971). With this modified medium, a 16 hr culture reached a pH of 7.6 to 7.8 and yielded a Sharples sediment of about 90 mg/15 L following inactivation with Cetavlon.

In the studies leading to the production of the Lot PA-3 preparation, a considerable loss of protein occurred during the various processing steps if the product was taken to dryness by washing with ethanol and acetone. Furthermore, after initial extraction of the Sharples paste with 1 M CaCl $_2\cdot 2H_2O$ , it was necessary to carry out further precipitations only with a Sodium salt, since the calcium salt tended to split part of the protein away from the complex. In addition, in order to assure that the purified protein-polysaccharide complex would pass through a 0.2  $\mu m$  filter, it was necessary to first freezedry it in an aqueous solution. The freeze-dried material was then weighed, dissolved in water at a concentration of 1 mg/ml, and the final sterile filtration was accomplished without difficulty.

Lot PA-3 was processed in the following manner, using 60 gm portions of Sharples paste. The 16 hr cultures, in 15 L batches, were inactivated with Cetavlon, and the sediments collected by Sharples centrifugation were extracted with 1 M CaCl .. 2H .. 0. After removal of DNA with a final concentration of 25% ethanól, the crude protein-polysaccharide complex was precipitated with ethanol. Processing from batch to batch was reproducible in the end result only if 60 gm portions of the Sharples paste were processed separately to final products. The parts can then be combined for the final sterile filtration. The crude precipitate was washed with ethanol and dissolved in water at approximately 50 mg wet weight/ml. The turbid solution was dialysed against distilled water at 5°C for 24 hours and then precipitated by the addition of ethanol to a final concentration of 90% in the presence of sodium acetate. This precipitate was collected by centrifugation at 8000 x g for 10 min and washed four times with 200 ml ethanol to insure that all of the sodium acetate was removed, with the final centrifugation at 16,300 x g for 30 min to remove as much ethanol as possible. This precipitate was dissolved in water at about 25 mg wet weight/ml and freeze-dried.

The dry weight of each of the 3 parts to be pooled for the vaccine was determined, and a final pool was prepared at 1 mg/ml in distilled water. After sterile filtration, a sterile concentrated sodium chloride solution was added to produce a physiological solution of the purified protein-polysaccharide complex.

Briefly, the major differences in Lot WZ-2 preparation from Lot PA-3 were as follows:

- 1) The protein for the complex was derived from cultures inactivated with 0.5% phenol. These organisms, from which the protein was extracted, were collected by centrifugation through the Spinco B-XVI continuous flow rotor.
- The polysaccharide was derived from cultures inactivated by Cetaylon and by extraction of the Sharples sediment with calcium chloride.

3) The protein and polysaccharide were combined in equal proportions and were then chromatographed on a Sephadex G-100 column to separate the toxin from the complex.

Both products (PA-3 and WZ-2) were filled to provide the equivalent of 25 ml of vaccine containing 100  $\mu g$  protein/ml, and were freeze-dried. The results of in vivo and in vitro assays on the final products are summarized in Table 1.

TABLE 1

Assays on Group B Protein-Polysaccharide Vaccines

						Lot WZ-2	3.04	3.10	0.04	16.9	>100,000		יי פריי		
	Rabbit Pyrogenicity g/kg ug/kg on-Pyro. Pyro.	0.50	2.00	:								d for Use)	Ratio Protein/Polysaccharide	1:1.56	1:1
	Rabbit Py µg/kg Non-Pyro.	0.375	1.00	:		Lot PA-3	2.54	3.97	0.09	6.93	>100,000	(Rehydrate	Ratio		
l) Animal Tests	Mouse Toxicity )	+8.4	+9.2	+9.6	2) In Vitro Assays	poq	ry	Svennerholm	U. V. Spectroscopy	Direct Weight	G-200 Sephadex	Protein and Polysaccharide Content (Rehydrated for Use)	Polysaccharide uq/dose	66	62
	Mouse Guinea Pia Safety Safety (Average Weight Gain in Grams	+28	77+	+68		Method	Lowry	Sve	u.	Dir	G-2	3) Protein and	Protein µg/dose	63.5	61.0
	Mouse Safet <u>y</u> (Average Weig	+0.85	+1.6	+2.35		Assay (mg/vial)		Acid	Acid	olids	Molecular Weight		Dose ml	0.2	0.5
	Vaccine Lot	PA-3	WZ-2	Control		Assay (	Protein	Sialic Acid	Nucleic Acid	Total Solids	Molecul		Vaccine Lot	PA-3	WZ-2

These products will be tested for efficacy in humans in the near future.

- b. An evaluation of several growth media for use in the large-scale production and purification of polysaccharides derived from cultures of a group Y strain (Boshard 80Y) of  $\underline{\textbf{N}}$ . meningitidis was carried out during this period.
- The four media employed were 1) modified Franz medium, 2) modified Franz medium containing 4 times the normal phosphate concentration, 3) Franz medium with yeast extract dialysate and 4) modified Franz medium containing 3 times the normal casamino acids concentration and one-fifth the dextrose concentration. For this study, the purification procedures were worked out and standardized to give a consistent end product. The results are summarized in the following table.

TABLE II

Comparison of Results Obtained with N. Meningitidis

Strain 80Y in Different Growth Media

Processing Steps	Modified Franz Medium	Modified Franz with 4XPO <sub>4</sub>	Franz with Yeast Extract Dialysate	Modified Franz with 3XCA and 1/5XDextrose
pH of	5.1	6.3	5.3	8.0
Culture Sharples	2.53	4.6	4.6	6.07
Sediment (gm/L)	2.55			
Crude	56.4	132.6	60.1	89.2
Extract (mg/L)				
Pheno1	16.6	47.0	1.9	27.3
Extract (mg/L)				
1 Alcohol	13.9			13.7
Pptn. + High				
Speed Centrifuga-				
tion (mg/L)				
<pre>3 Alcohol Pptns + High</pre>	-	12.2		
Speed				
Centrifuga-				
tion (mg/L)				
In Vitro				
Assays				
Protein, (%)		0.19	26.2*	0.50
Nucleic Acid	1, 0.56	0.45	5.3*	0.61
Sialic Acid (%)	51.5	62.0	37.0*	45.9
Molecular				
	100,000	>100,000		
Pyrogeni- city	Non-pyrogen 2.5 μ/kg	0.25 μg/k	g	

\*Phenol Extract Product

The results indicated that the modified Franz medium gave a reasonable yield of chemically pure polysaccharide with less processing than was required for the modified Franz with 4X phosphate. Although the modified Franz with 3X Casamino Acids and 1/5 X dextrose medium gave a comparable yield, the sialic acid content was lower than postulated (50% or greater). Subsequent experiments with the modified Franz medium gave final yields of 14.5 and 16.0 mg/L of purified polysaccharide.

The crude polysaccharide obtained following calcium chloride extraction, DNA removal, precipitation, and drying was dissolved in a 1/10 dilution of saturated sodium acetate (pH 7.0) at 10 mg/ml. It was then fractionated 3 times using a 75% phenol solution and the phenol layers were washed sequentially with one volume of 1/10 saturated sodium acetate. The aqueous phases and the wash were combined, dialysed against distilled water at  $5^{\circ}$  C, precipitated with 80% ethanol in the presence of calcium, and the precipitate dissolved in 0.02 M CaCl<sub>2</sub>. This solution was made opalescent by using a final concentration of 33% ethanol and was centrifuged at 100,000 x g for 4 hrs. The supernatant was then precipitated using 80% ethanol in the presence of sodium acetate.

It had been suggested by a colleague that one fractionation with 90% phenol solution and elimination of the wash of the phenol layers would yield a product with the same degree of purification as the three 75% phenol fractionations described above. This simplified procedure was tried and followed with one ethanol treatment and a high speed centrifugation. A comparison of the two products is given in the table below.

TABLE III

Comparison of 90% and 75% Phenol Fractionation Procedures

	90% Phenol (1 Extr No Wash	75% Phenol ) (3 Extr. + Wash)
Yield (mg)	31.2	13.9
Protein (%)	0.86	0.48
Nucleic Acid (%)	0.68	0.56
Sialic Adic (%)	56.5	51.5
Molecular Weight	>100,000	>100,000
Pyrogenicity	Pyrogenic at	Non-Pyrogenic at
		2.5 μg/kg

It can be seen that the yield is considerably greater with the 90% phenol fractionation; however, this product proved to be pyrogenic at the same level at which the other was non-pyrogenic. This factor would likely cause more side reactions in humans and a less toxic vaccine is to be preferred.

Some difficulty has been experienced in sterilizing the final product solution by passage through a 0.2  $\mu m$  filter. Attempts are currently being made to eliminate this problem so that a production lot of the 80Y strain polysaccharide can be prepared for evaluation of immunogenicity in man.

c. An experimental study on the development of a purified polysaccharide derived from cultures of Strain 29E of N. meningitidis was initiated at the request of the Department of Bacterial Diseases, WRAIR. Studies similar to those described above with the 80Y strain were carried out. Results obtained from 15 L culture batches in 3 different media are summarized in the following table.

TABLE IV

Comparison of Results Obtained with N. Meningitidis

Strain 29E in Different Growth Media

Processing Steps	Modified Franz Medium	Modified Franz with 4XPO <sub>4</sub>	Modified Franz with 3XCA and 1/5 X Dextrose
pH of Culture Sharples Sediment (gm/L) Crude Extract (mg/L) Phenol Extract (mg/L) l Alcohol Pptn + High Speed Centrifugation (mg/L)	5.5 3.1 68.0 22.0 16.0	6.5 5.7 85.8 12.2 11.9	8.2 5.9 42.8 11.8 11.9
In Vitro Assays			
Protein (%) Nucleic Acid (%) Keto-Deoxyoctonic Acid (%) Molecular Weight Pyrogenicity	0.93 0.63 75.7 >100,000 Pyrogenic at 2.5 µg/k	1.81 1.17 76.0  	1.53 3.19 74.0 
	Non-Pyrogen at 0.25 µg/		

Again, the modified Franz medium was the medium of choice with regard to ease of processing to a purified product. A production lot was therefore processed in the same manner as described above for the 80Y strain, except that it was necessary to include a high speed clarification centrifugation before proceeding to the alcohol treatment. This product was sterilized by filtration, filled at 2.5 mg polysaccharide per vial, and freeze-dried. It is being held for testing in man until the final product sterility, safety, toxicity, and pyrogenicity assays are completed. In vitro assays of the bulk product are shown in the following table.

### TABLE V

# In Vitro Assays of N. Meningitidis Strain 29E Polysaccharide

Protein (%)	0.61
Nucleic Acid (%)	0.97
Keto-Deoxyoctonic Acid (%)	78.1
Carbohydrate (%)	22.8
Molecular Weight	>100,000
Pyrogenicity	Non-Pyrogenic
	at 0.25 μg/kg

d. Since both the 80Y and 29E meningococcal strains grew well in the modified Franz medium and required a minimum of processing to produce a purified product, the 135-III strain of N. meningitidis was also grown in this medium. A 16 hr culture had a pH of 5.2, and a Sharples sediment of about 80 gms/15 L batch was obtained. After fractionation with 75% phenol, the protein and nucleic acid content were each about 3% with this strain; however, it was found that high speed centrifugation alone, or combined with ethanol opalescence, caused a considerable loss of the polysaccharide with little reduction in the protein and nucleic acid content. The sialic acid content was about 50% and carbohydrate about 40%. The molecular weight of the polysaccharide was greater than 100,000. Studies with this strain are continuing in an attempt to find a satisfactory method to produce a purified polysaccharide which will be a suitable immunogen for human use.

### 2. Plague Vaccine.

Work has continued on a collaborative study with the Department of Hazardous Microorganisms, WRAIR, to determine whether an attenuated strain of Yersinia pestis, inactivated with formalin, could replace the highly hazardous virulent strain currently used in the preparation of the U.S. Army plague vaccine.

In previous studies (Annual Report, 1976), the attenuated strains were grown on Bacto Blood Agar Base (Difco) and it was

determined that 0.05% formalin was sufficient to completely inactivate the harvested cultures within 24 hours. In the current study, the attenuated strain (Al122) was grown on a modification of "E" Medium, a medium free of blood group substances which is used in the commercial production of plague vaccine, and the resulting harvest pool was divided into 2 portions. One portion was inactivated with 0.05% formalin and the other with 0.65% formalin (the concentration of formalin used to inactivate the virulent strain of  $\underline{Y}$ . pestis for the commercial production of plague vaccine). The two portions were held at room temperature, with frequent shaking and samples were withdrawn at designated time periods to determine rates of inactivation. The results are summarized in Table VI.

TABLE VI

# Formalin Inactivation of Yersinia Pestis (strain All22) Harvest

Formalin (%)	Inactivation Time (Hrs.)			
	24	48		
0.05	*			
0.65	<del></del>			

<sup>\*</sup>No apparent growth on agar.

At both formalin levels the harvest pools were completely inactivated within 24 hours. Nitrogen assays on these two inactivated preparations indicated that they contained approximately three times the nitrogen present in the standard U.S. Army plague vaccine as is shown in Table VII.

TABLE VII
Nitrogen Assays on Inactivated Plague Preparations

Preparation	Nitrogen (mg/ml)
A1122-0.05% Formalin	1.25
A1122-0.65% Formalin	1.12
U.S. Army Vaccine	0.39

These preparations have been submitted to the Department of Hazardous Microorganisms, WRAIR, for evaluation in animals. The antibody responses and protective activities of the experimental vaccines prepared from the attenuated strain will be compared with those of the standard vaccine on an equivalent nitrogen basis.

# 3. Dengue Virus Vaccines.

During this period investigations directed towards the development of a dengue virus type 2 (DEN-2) vaccine for human use were continued. Studies were also carried out on the development of a mouse potency assay for the evaluation of DEN-2 vaccines. In addition, an attempt to adapt dengue virus type 3 (DEN-3) to grow in a certified diploid strain of fetal rhesus monkey lung (DBS-FRhL-2) cells was initiated.

a. Using procedures outlined previously (Annual Reports 1974, 1975 and 1976), several lots of an inactivated DEN-2 vaccine were produced and tested in animals. Preliminary results indicated that the serological conversion of mice and guinea pigs was quite inconsistent, and three doses of the vaccine appeared to be required for significant serological conversion. It was also observed that the infectious virus titers of the harvest fluids prior to inactivation varied considerably, although the infectious fluids were harvested on the similar basis of time and cytopathogenic effect (CPE). This variation in infectious titer could account for the large variability in the immunologic responses of the animals. Therefore, the hemagglutination (HA) of goose red blood cells by the culture fluids was investigated as an additional criterion for the harvesting of infectious fluids.

In a previous study (Annual Report, 1976), growth curves were obtained with three different concentrations of virus particles to determine the relationship between the number of virus particles in the inoculum and optimal time of harvest. This experiment was repeated, and the HA activity of the infectious fluid was also determined.

Confluent monolayers of DBS-FRhL-2 cells in 75 cm<sup>2</sup> flasks were washed with 25 ml of Hank's Balanced Salt Solution (HBSS) and then inoculated with DEN-2 seed material (Annual Report, 1974) at a multiplicity of infection (MOI) of 1, 0.1 and 0.01. Virus was adsorbed at 35°C for 90 minutes, followed by removal of the inoculum and three rinses of the cell monolayers with 25 ml of HBSS. After the addition of 25 ml of maintenance medium (Eagle's Minimum Essential Medium with antibiotics but no serum additives), the flasks were incubated at 35°C. At 1, 2, 3, 4, 5, 6, 7 and 8 days post-inoculation, 0.3 ml samples from each of two flasks were removed, mixed with an equal volume of inactivated fetal bovine serum (FBS) and frozen at -70°C. Samples were assayed by plaquing in LLC-MK<sub>2</sub> cell monolayers. In addition, 0.2 ml samples from each of two flasks were removed and immediately tested for HA. The results are presented in Table VIII.

TABLE VIII

Effect of Viral Concentration of Inoculum on Virus Titer and Hemagglutination Activity of Dengue Virus Type 2

	MOI o		MOI of C	1.1	MOI of 0.	01
Day	Titera	HAB	Titer	HA	Titer	HA
1	1.0×10 <sup>5</sup>	0	3.1×10 <sup>4</sup>	0	2.3×10 <sup>3</sup>	0
2	1.1×10 <sup>5</sup>	0	3.8×10 <sup>4</sup>	0	9.0x10 <sup>3</sup>	0
3	2.1x10 <sup>5</sup>	2	1.2×10 <sup>5</sup>	0	5.0×10 <sup>4</sup>	0
4	4.5×10 <sup>5</sup>	4	4.1×10 <sup>5</sup>	0	6.5×10 <sup>4</sup>	0
5	1.1×10 <sup>6</sup>	8	8.1×10 <sup>5</sup>	0	1.0x10 <sup>5</sup>	0
6	1.4×10 <sup>6</sup>	16	1.2×10 <sup>6</sup>	2	6.0×10 <sup>4</sup>	0
7	3.0×10 <sup>6</sup>	16	1.3×10 <sup>6</sup>	4	3.0x10 <sup>5</sup>	0

- a. PFU/0.2 ml
- b. Per 0.5 ml

As can be seen from the above table, maximum HA titers were achieved with an MOI of 1. It also appears that HA can be used as an additional criterion for the harvesting of infectious fluids.

It was also of interest to determine what effect serum additives would have on the HA of DEN-2 virus, and at the same time to check the reproducibility of the experiment in terms of HA. Using an MOI of I and the same procedures as previously described, the above study was repeated with and without the addition of Human Serum Albumin (HSA) and Fetal Bovine Serum (FBS) to the maintenance medium. The results are shown in the following table.

TABLE IX

Effect of Different Serum Additives on the Hemagglutination Activity of Dengue Virus Type 2

	No Serum Ad	ditive	HSA		FBS	
Day	<u>Titer</u> a	HA b	Titer	HA	Titer	HA
1	3.4×10 <sup>4</sup>	0	1.5×10 <sup>4</sup>	0	3.6×10 <sup>4</sup>	0
2	1.6×10 <sup>5</sup>	0	1.9×10 <sup>5</sup>	0	3.8×10 <sup>5</sup>	0
3	5.5×10 <sup>5</sup>	2	2.2x10 <sup>5</sup>	0	1.4×10 <sup>6</sup>	0
4	9.4×10 <sup>5</sup>	4	2.1×10 <sup>5</sup>	0	1.9×10 <sup>6</sup>	0
5	9.7×10 <sup>5</sup>	4	5.1×10 <sup>5</sup>	0	2.0×10 <sup>6</sup>	0
6	1.5×10 <sup>6</sup>	8	1.4×10 <sup>6</sup>	2	2.5×10 <sup>6</sup>	0
7	1.0×10 <sup>6</sup>	16	2.0×10 <sup>6</sup>	4	2.4×10 <sup>6</sup>	0

a. PFU/0.2 ml

b. Per 0.05 ml

The serum additives did have an inhibitory effect on the HA titers, as indicated in the above table. However, the virus and HA titers of the infectious fluid containing no serum additive were comparable to those in the previous experiment. It thus appears that the HA titer would be a good indicator for the harvest of infectious fluids.

On the basis of the above results, an HA titer of 16 was selected as the minimum titer for the harvesting of infectious fluids. Based on this criterion, an inactivated DEN-2 vaccine was made using previously described methods. Rabbits were immunized with varying doses of the vaccine to reevaluate the number of doses required for complete serological conversion. Three rabbits received 1 ml intramuscularly (IM) on day 0, three other rabbits received 1 ml IM on days 0 and 7, and the last three rabbits received 1 ml IM on days 0, 7 and 21. All rabbits were bled on day 28. The results of the hemagglutination-inhibition titrations on the rabbit antisera are presented in the following table.

TABLE X

Effect of Number of Doses on NI Responses of Rabbits
Immunized with an Inactivated DEN-2 Vaccine

Rabbit	No. of Doses	H <b>1</b> -Titer*
1	1	0
2	1	0
3	1	0
4	2	20
5	2	0
6	2	10
7	3	40
8	3	40
9	3	40

\*Reciprocal of the HI antibody against 4-8 units of DEN-2 virus HA

These results substantiate the previous observation that three doses of vaccine are required for significant serological conversion. However, whether the harvesting of infectious fluid based on an HA titer of 16 will eliminate some of the variability that was encountered with previous vaccines is still to be determined.

b. In the process of trying to increase the antigenic mass of a DEN-2 vaccine, a precipitate was unexpectedly formed when a freezedried product was resuspended with water to 1/10 of its original fluid volume. It was determined that this precipitate contained the bulk of the live virus and the HA activity. During further investigations, this precipitate was easily produced by adding magnesium and sodium salts to infected cell culture fluids. Since this appeared to be a simple method of preparing concentrated hemagglutinins of DEN-2 virus, further investigations were carried out.

Confluent monolayers of DBS-FRhL-2 cells were inoculated as previously described. When 4+ CPE developed, the fluids were harvested and clarified by centrifugation at 2500 x g for 10 min at  $^{4}$  C. Various concentrations of Mg-Na salts were added to the virus suspensions and then the fluids were adjusted to pH 8.0, 8.5 and 9.0 with IM NaOH. After 1 hr at  $^{4}$  C, the precipitates were sedimented at 2500 x g for 10 min at  $^{4}$  C, the fluids were decanted and drained, and the pellets were resuspended to 1/10 volume in borate-buffered-saline (pH 9.0) containing 0.2% bovine serum albumin. The results are presented in Table XI.

TABLE XI

Recovery of HA and Virus Titers of DEN-2 After Precipitation
With Salts at Different Concentrations and pH

Salts*	рН	HA(Per 0.05 ml)	Titer (PFU/0.2 ml)
3X	8.0	128	9.0x106
	8.5	128	7.0×107
	9.0	256	1.7×107
5X	8.0	128	1.5×107
	8.5	256	1.5×10/
	9.0	128	9.0x10
7X	8.0	64	7.0×105
	8.5	256	1.4×10/
	9.0	256	9.0x106
9X	8.0	64	8.0x106
	8.5	128	1.1x106
	9.0	128	9.0x106
Original fluid (1X)	7.0-7.2	32	1.3×10°

\*MgSO $_4\cdot 7H_2O$  and NaH $_2PO_4\cdot H_2O$  are found in Earl's Minimum Essential Medium in the following concentrations: MgSO $_4\cdot 7H_2O$ =200 mg/liter; NaH $_2PO_4\cdot H_2O$ =140 mg/liter. In the above table, therefore 3X indicates 3 times the concentration of both salts.

On the basis of HA and infectious titer, comparable results were obtained with the various concentrations of salts and pH. However, as the salt concentration was increased, there was an increase in the amount of precipitate without an increase in HA or infectious titer. For this reason, a lower salt concentration is desirable. Therefore, subsequent studies were done using a 3x salt concentration at pH 9.0. When the infectious tissue culture fluid was concentrated 10 fold, the efficiency of recovery was approximately 100% in terms of both HA and infectious titer. However, the efficiency of recovery decreases drastically when the infectious fluid was concentrated 50 fold and decreases to a completely unprofitable level when concentrated 100 fold.

This salt precipitated DEN-2 antigen gave similar HA patterns and HI antibody titers when compared to HA antigen prepared by sucrose-acetone extraction of suckling mouse brain pools. The HA activity remained stable for at least 6 months at  $^{40}$  C.

Precipitation of tissue culture propagated DEN-2 virus with salts may also provide a simple method for concentrating live virus for vaccine preparation. Additional studies are planned to determine the usefulness of this procedure.

c. A mouse potency assay for the evaluation of DEN-2 vaccines is currently being investigated. Several strains of DEN-2 virus were titrated intracerebrally (IC) in adult mice to determine their potential for use as a challenge virus. One particular strain, WP-131, gave very promising results. The following table shows the titration of this virus and also demonstrates the death pattern, which is fairly clean and unusual for dengue.

TABLE XII

Titration of DEN-2 Virus (Strain WP 131) in Adult Mice

Day	10-1	10-2	10-3	10-4	10-5
1	0/5*	0/5	0/5	0/5	0/5
10 11 12 13 14 15 16 17 18 19 20 21	2/5 3/5 3/5 5/5	1/5 1/5 2/5 2/5 2/5 4/5 4/5 5/5	0/5 0/5 0/5 0/5 1/5 2/5 2/5 5/5	0/5 0/5 0/5 0/5 0/5 2/5 3/5 3/5 3/5 4/5	0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5

Each mouse was inoculated IC with 0.05 ml

\*No. of deaths/total inoculated

From the above results, it can be observed that the death pattern demonstrates a graded dose response. Thus it appears that this strain of DEN-2 virus may be useful as a challenge virus in the mouse potency test.

In a preliminary potency assay, groups of mice that were inoculated with either a formalin inactivated DEN-2 vaccine or a live attenuated DEN-2 vaccine were protected against a challenge of approximately 1000 LD $_{50}$  doses of the WP 131 strain. Additional studies are currently being carried out to further determine the usefulness of this mouse assay for the evaluation of the immunogenicity of dengue vaccines.

d. Previous attempts to adapt DEN-2 virus to chick fibroblast cell cultures were unsuccessful (Annual Report, 1974). Another attempt

was initiated during this period, using a totally different approach. Embryonated chick eggs were inoculated intravenously with DEN-2 virus and a series of embryos were then bled on successive days for 10 days. The results of this viremia study in embryonated chick eggs is presented in the following table.

TABLE XIII

Dengue Type 2 Viremia in Embryonated Chick Eggs

Day of Bleeding	Titer (PFU/0.2 ml)
1	0
2	0
3	1
4	0
5	5.5
6	1.5
7	5
8	2
9	3
10	1

The day 5 and 7 bleedings were further passaged intravenvenously in embryonated chick eggs without any success. The day 9 material was then passaged into chick fibroblast cell cultures. The following table shows the results of the first four passages.

TABLE XIV

Passages of Dengue Type 2 (PR 159) in Chick Fibroblast Cells

Day of Harvest	Titer (PFU/0.2 ml)
5	2.2×10 <sup>1</sup> 1.3×10 <sup>2</sup> 2.0×10 <sup>3</sup> 1.4×10 <sup>2</sup>
4	1.3×10 <sup>2</sup>
3	2.0×103
3	1.4×10 <sup>2</sup>
	Day of Harvest  5 4 3 3

Further passages will be carried out in chick fibroblast cultures in an attempt to increase the yield of infectious virus to a level satisfactory for vaccine production.

e. Passage of dengue virus type 3, strain CH 53489 (DEN-3), in a certified diploid strain of fetal rhesus monkey lung (DBS-FRhL-2) cells was continued. Ten additional passages were made during this period, using the procedures previously described (Annual Report, 1976). Since only one passage was done in 1976, the entire passage history of DEN-3 in certified DBS-FRhL-2 cells is presented in the following table.

TABLE XV

Passages of DEN-3 Virus in Certified DBS-FRhL-2 Cells

Passage	Inoculum	Day of Harve	est Titer (PFU/0.2 ml)
1	3.8×10 <sup>2</sup>	7*	2.0×10 <sup>0</sup>
	(human serum)	14	2.5×10
2	(human serum) 6.2x10 <sup>2</sup>	3*	0 ,
		4*	2.0×10
		5*	6.0×10
		6*	8.7×10 <sup>2</sup>
		7	1.3×10 <sup>3</sup>
3	1.6x10 <sup>3</sup>	3*	1.0×10,
		7	1.3×10 <sup>2</sup>
4	1.6×10 <sup>2</sup>	3*	ND 0
		7	5.2×10 <sup>2</sup>
5	6.5x10 <sup>2</sup>	7 3* 7 3* 7	0
		7	5.3×10 <sup>2</sup>
6	1.3x10 <sup>3</sup>		1.5x10 <sup>0</sup>
		7	1.5×10 <sup>0</sup> 7.4×10 <sup>0</sup>
7	1.8x10 <sup>3</sup>	3*	8.0×100
	1.02.10	ý	9.1×10 <sup>2</sup>
8	2.3×10 <sup>3</sup>	3* 7 3* 7 3*	7.0×100
		7	1.4×101
9	3.5×10 <sup>3</sup>	3*	6.0×103
		7	1.6×103
10	4.0×10 <sup>3</sup>	3*	ND ,
		7	1.4×104
11	3.5×10 <sup>4</sup>	7 3* 7 3* 7	1 2×103
	3.5010	7	1.2×10 <sup>3</sup> 1.0×10 <sup>4</sup>
*Culture	fluids harvested an	d call sheets	refed with FMFM+29 FRS

\*Culture fluids harvested and cell sheets refed with EMEM+2% FBS

At the passage II level, a portion of the 7 day harvest was prepared for further studies. The continuous passages of the original DEN-3 human serum isolate in these DBS-FRhL-2 cells yielded DEN-3 virus that produced only small plaques in LLC-MK2 cells and exhibited temperature sensitive characteristics. Monkey inoculation experiments, however, indicated that this virus still produced viremia.

Currently, the passage 11 materia, is being cloned by a terminal dilution technique, since a plaque assay of DEN-3 virus in these certified cells is not yet possible. Each clone will then be characterized and evaluated as a candidate seed strain for use in the development of a DEN-3 vaccine.

# 4. Diagnostic Antigens and Antisera.

Previous studies in this laboratory have demonstrated the

feasibility of preparing freeze-dried and tableted bacterial antigens and antisera for use in diagnostic tests for the febrile diseases (Annual Report, 1976; Berman et al., 1977). During this period am evaluation of the stability of the tableted antigen preparations was initiated.

Tableted preparations of five febrile antigens, sealed in vacuo, were placed in storage at  $^4$  C,  $^3$  C,  $^4$  C and  $^5$  C. At three month intervals, sample tablets were removed from incubation, rehydrated with 20 ml phenolized distilled water, and the resulting antigen suspensions were tested for agglutinability in dilutions of homologous antisera by the standard tube-type agglutination test.

Similar results were obtained over a nine month period (the maximum time tested thus far) with tablets stored at all four temperatures. The titers obtained with the  $56^{\circ}$  C samples are summarized in the table below.

TABLE XVI Stability of Tableted Febrile Antigen Preparations Stored at  $56^{\circ}$  C

Antigen		Time (months)				
	0	3	6	9		
S. typhi "0"	640*	320	160	320		
S. typhi "H"	1280	320	640	1280		
S. paratyphi "H"	640	640	640	320		
S. schottmuelleri "H"	640	640	640	320		
Proteus 0X-K	320	80	80	80		

<sup>\*</sup>Reciprocal of agglutination titer of homologous antiserum.

With all of the antigen preparations, in the tests performed at the different time periods, the differences in the serum titers obtained were not more than 4-fold, and are therefore not considered to be significant. Thus, after storage at temperatures up to  $56^{\circ}$  for a period of 9 months, there appears to be no significant loss in the ability of the tableted antigens to react with the homologous antibody to titer.

# 5. Vaccine Potency Assays.

During the past year studies have continued in an effort to determine whether the addition of iron to sub-standard lots of hog gastric mucin would sufficiently enhance the virulence of <u>Salmonella typhi</u>, strain Ty2, to permit its use as a challenge organism in mouse potency assays for the evaluation of typhoid vaccines.

Previous investigations have shown that, with the addition of varying concentrations of iron in the form of ferric ammonium citrate to several different lots of mucin, an enhancing effect of iron on virulence could be demonstrated (Annual Report, 1975). In addition, mouse protection test assays were performed to determine the efficacy of U.S. Standard typhoid vaccine, Lot #6A, by challenging previously immunized mice with the Ty2 strain suspended in 5% mucin alone and in 5% mucin supplemented with iron. Additional assays compared U.S. Standard typhoid vaccine, Lot #6A, with a commercially prepared typhoid vaccine involving the same two challenges. In the first of these experiments the results showed no significant change in ED<sub>50</sub> value for vaccine Lot #6A and in the latter experiment, although there was a 2-fold difference between the ED<sub>50</sub> values obtained with the 2 challenge suspensions, the relative potencies of the 2 vaccines were identical (Annual Report, 1976).

At this point, the study was temporarily halted because of an infection in the Walter Reed mouse colony necessitating the destruction of the colony. A new colony of mice was eventually produced and preliminary virulence titrations revealed that the newly formed Walter Reed ICR mouse strain was considerably more resistant to typhoid strain Ty2 when tested with the available mucin lots. A search for a more suitable mouse strain was therefore undertaken.

Two strains of mice were obtained from the National Institutes of Health (NIH) and were compared with the new Walter Reed ICR strain for suitability. Using mucin lot #0347A001, which had previously been demonstrated to be satisfactory, the results as shown in Table XVII revealed that the NIH Swiss males provided the lowest LD<sub>50</sub> value (5.0 organisms), well below the 10 colony forming units required by the U.S. Food and Drug Administration for typhoid challenges (Code of Federal Regulations, 1976).

#### TABLE XVII

Virulence Titration of <u>S</u>. <u>Typhi</u> Ty2 (in 5% Mucin<sup>\*</sup>) In Various Mouse Strains

Mouse Strain	LD <sub>50</sub> **	
NIH Swiss Males	5.0 or	ganisms
NIH General Purpose Males	13.5	11
W. Reed ICR Males	80.0	11
NIH General Purpose Females	125.0	11
W. Reed ICR Females	1125	H

\*Mucin Lot #0347A001

\*\*Calculated by the Miller-Tainter Probit Method

It was desirable therefore to determine the extent of virulence enhancement, if any, that could be achieved by the addition of iron, using the NIH Swiss male mice. Two lots of mucin previously found to be substandard in typhoid virulence titrations were compared with the known satisfactory lot #0347A001. Various concentrations of iron were added to suspensions of the sub-standard mucin preparations and mouse virulence titrations were performed. The results of this experiment are shown in Table XVIII.

### TABLE XVIII

The Effect of Iron on the Virulence of S. Typhi Strain Ty2 for Mice\*

Mucin	Iron Concentration (mg/kg mouse body weig	uht) LD 50**
#0347A001	0	4.2 organisms
#96181	0	90.0
11	10	6.5 "
H	15	7.5 "
- H	20	5.2 "
#1715357	18	4320.0 "
H		13.5
	15	12.0 "
н	20	10.5 "

\*Mouse strain - NIH Swiss Males

\*\*Calculated by the Miller-Tainter Probit Method

The results, as had been demonstrated with the formerly used Walter Reed ICR strain, clearly showed that the addition of iron to mucin preparations produced an enhancing effect on virulence, and further, that different mucin lots require different amounts of iron to reduce the LD<sub>50</sub> value to an acceptable level (not more than 10 colony forming units).

Additional virulence titrations were performed in order to compare the LD<sub>50</sub> values of the Ty2 strain suspended in three different mucin lots. These mucin lots were supplemented with varying concentrations of iron and subsequently inoculated into both the NIH and Walter Reed strains of mice. These results are summarized in Table XIX.

TABLE XIX

Comparison of the Virulences of <u>S</u>. <u>Typhi</u> Ty2 With Different Lots of Mucin and Different Strains of Mice

# Mucin Lot #0347A001

Iron* Added	NIH Swiss M	lales	NIH General	Purpose Males	W. Reed Males
0	~10.0**	Org.	25.0	0rg.	350 Org. 893 ''
5 mg/kg			4.5	11	893 ''
10 mg/kg			2.7	u	52.0 "
15 mg/kg			3.0	11	7.5 "

### Mucin Lot #96181

	NIH Swiss	Males	NIH General	Purpose	Males	W.	Reed	Males
0	80.0	Org.	(not do	ne)		~20	000	Org.
5 mg/kg	15.0	11						
10 mg/kg	6.0	- 11				>1	5000	- 11
15 mg/kg	8.0	11						
20 mg/kg	0.6	11				>1	5000	11
30 mg/kg						•	>500	11
40 mg/kg							350	- 11

# Mucin Lot #1715357

	NIH Swiss Males	NIH General Purpose Males	W. Reed Males
0	~30,000 Org.	>30,000 Org.	~30,000 Org.
10 mg/kg	>300	54.0 "	300 ''
15 mg/kg	~15.0 "	100.0 "	
20 mg/kg	~ 7.5 "	60.0 "	>300 ''
30 mg/kg			>300 ''

\*Iron=Ferric Ammonium Citrate \*\*LD<sub>50</sub> Value

Again the results showed that with the NIH Swiss male mice, the addition of increasing concentrations of iron to the mucin suspensions did reduce the LD values to 10 organisms or less with each of the three mucin lots. However, with the Walter Reed ICR males and NIH General Purpose Males, addition of iron concentrations even up to 30 mg/kg did not sufficiently reduce the LD values with mucin lots #96181 and #1715357. These two mucin lots had been considered sub-standard in previous titrations without the addition of iron. Higher concentrations of iron were not used because it has been observed that ferric ammonium citrate is generally toxic for mice at the concentration of 60 mg/kg or

above. With mucin lot  $\#0347A00^{1}$ , in the NIH General Purpose Males and Walter Reed ICR males, the LD values were reduced to less than 10 organisms with concentrations of 5 and 15 mg/kg of iron, respectively.

Thus it appears that the NIH Swiss male mice are the most satisfactory of the several strains tested. This strain of mice is currently being used to determine whether iron-supplemented mucin preparations can be employed in the mouse protection test to evaluate typhoid vaccines. The usefulness of iron-supplemented mucin in mouse potency assays for cholera and meningococcal vaccines will also be evaluated.

Project 3M161102BS01 BASIC RESEARCH ON MILITARY DISEASES

Work Unit 136 Development of biological products

### Literature Cited.

### References.

- 1. Berman, S., Vincent, G., Altieri, P.L., and Lowenthal, J.P.: Diagnostic Antigens and Antisera in Tablet Form. Journ. Clin. Microbiol.  $\underline{5}$ : 492-3, 1977.
- Code of Federal Regulations, Title 21: Food and Drugs, sec.
   Revised as of Apr. 1, 1976.

# Publications.

- 1. Altieri, P.L., Berman, S. and Lowenthal, J.P., Sublimation of Formaldehyde in Freeze-Drying. Developments in Biological Standardization. International Symposium on Freeze-Drying of Biological Products 36: 231-6, 1977.
- 2. Zollenger, W.D., Mandrell, R.E., Berman, S., Altieri, P.L., Lowenthal, J.P. and Artenstein, M.S. Preliminary Testing of a Meningococcal Type II Protein Vaccine in Animals and Laboratory Volunteers. 16th Interscience Conference on Antimicrobial Agents and Chemotherapy. Abstract #93, 1976.
- 3. Berman, S., Vincent, G., Altieri, P.L., and Lowenthal, J.P., Diagnostic Antigens and Antisera in Tablet Form. Journ. Clin. Microbiol.,  $\underline{5}$ : 492-3, 1977.
- 4. Dubois, D.R., Berman, S., Rourke, S.M., Timchak, R.L. and Lowenthal, J.P. Cultivation of Dengue Virus Type 2 in Candidate Substrates for Vaccine Production. Journ. Biol. Standardization  $\underline{5}$ : (in press), 1977.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY			DA (	OB 6537	77 10	01	DD-DR	&E(AR)636	
3. DATE PREV SUMPRY	4. KIND OF SUMMARY	S. SUMMARY SCTY	. WORK SECURITY					DATA-	LEVEL OF SUM
76 10 01	D. Change	U	U	_ N	Α _	NL		□ MO	A WORK UNIT
10. NO./CODES:*	PROGRAM ELEMENT	PROJECT		TASK AREA NUMBER WORK UNIT NUMBER					
& PRIMARY	61102A	3M161102B	S01		00		137		
b. CONTRIBUTING									
c. c)@0@@0@XX	Cards 114F								
	Security Classification Code	•							
(U) Pathol	ogic Manifest	ations of	Zoonotic Di	Lsease	es of Mil	itary .	Importan	ce	
002600 Bio	logy	14. ESTIMATED COM	PLETION DATE	IS FUND	DING AGENCY		16. PERFOR	MANCE WETH	100
74 02		Cont.		D	A	1	C. TI	n-House	a
17. CONTRACT/GRANT				_	OURCES ESTIMAT	E & PROFE	SSIONAL MAN Y		DS (In thousands)
A DATES/EFFECTIVE:		EXPIRATION:			PRECEDING				
b. NUMBER:*	NA			FISCAL	77		8		147
C TYPE:		& AMOUNT:		YEAR	CURRENT				
& KIND OF AWARD:		f. CUM. AMT.			78		8		212
19. RESPONSIBLE DOD	ORGANIZATION			20. PER	FORMING ORGANI	MOITA			
MAME: Walter	Reed Army Ins	titute of	Research	NAME:*	Walter F	Reed Arr	my Insti	tute o	f Research
					Divisio Washin		-		
ADDRESS: Wash	ington, DC 20	012		LOURES	washin	igcon, i	20012		
				PRINCIP	AL INVESTIGATO	R (Furnish SSA	N II U.S. Academ	ic Inelitution)	
RESPONSIBLE INDIVID	141				JOHNSON				
	ND, GARRISON,	COT				2-576-2		D. V.PI.	
TELEPHONE: 202		COL		1	SECURITY ACCO				
21. GENERAL USE	370-3331			-	TE INVESTIGATO				
				NAME:	REARDON	. M. J	., SEELY	. J. C	
Foreign int	elligence not	considere	d	NAME:	KEENAN,				
22. KEYWORDS (Frecede	BACH with Society Cleanit		Wildlife;						
(U) Electro	n Microscopy;	The same of the sa			and the same of th		-		U) Brain;
	TIVE. 24 APPROACH, 25			lentified by	number. Precede f	ozt of each wife	h Socurity Cloself	tcettan Code.	
23 (U) To s	tudy and defi	ne pathoge	nesis of sp	conta	neous and	exper	imental	trypano	osomiasis,
	is, schistoso								
study spont	aneous severe	renal dis	ease in col	loniz	ed and qu	aranti	ned aotu	s monke	eys.
Provide ana	tomic patholo	ay support	for wildl:	ife e	pidemiolo	gical :	surveys	in the	Trans-
Amazon and	other militar	y installa	tions. All	l pro	jects und	ier stud	dy are o	f mili	tary im-
portance as	zoonotic dis	eases or a	nimal mode:	ls fo	r human d	lisease	s.		1000
24 (U) Stud	ies will util	ize conven	tional gros	ss and	d histopa	tholog	y, clini	cal pa	thology,
electron mi	croscopy, his	tochemistr	y, immunoh:	istoc	hemistry	and rad	dioisoto	pic te	chniques.
25 (U) 76 1	0-77-09 The	pathogenes	is of exper	rimen	tal Leish	mania d	donovani	infec	tion in
the cynomol	gus monkey, h	amster, my	stromys and	d dog	is curre	ently b	eing def	ined.	The
	s of experime								
studied. T	he role of di	sseminated	intravascu	ular	coaqulati	on in	the path	ogenes.	is of
	rhodesiense								
	ogy studies o								
	e are in proq								
	s in tissues								
	phritis induc								
	. A study to								
	ocystis fusif								
	Beltsville, M								
	fusiformis h								
	data compile								
	eing continue								
Army Instit	ute of Resear	ch Annual	Progress Re	eport	, 1 Jul 7	6-30 S	ep 77.		
A veilable to contract	tors upon originator's appr	ovel	,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,						

Project 3M161102BS01 MILITARY INTERNAL MEDICINE

Task 00 Military Internal Medicine

Work Unit 137 Pathologic Manifestations of Zoonotic Diseases of Military Importance

Investigators

Principal: Anthony J. Johnson, MAJ, VC

Associates: MAJ Michael Reardon, MAJ Ralph Bunte, CPT Kevin Keenan, CPT

LuAnn McKinney, CPT Charlotte Miller, CPT John Seely, CPT

Carol Thompson and CPT Henry Wall

### Background

To diagnose, define, investigate, compare and analyze known and potential communicable diseases common to both man and animal. Special emphasis will be focused on control and protective methods against the occurrence and tissue injury induced by these diseases. A continual recognition will be given to new animal models for study of human diseases. A major effort will be directed toward defining the mechanistic events and reactions at the cellular levels occurring in the development of these diseases. Gross pathology, histopathology, clinical pathology, comparative pathology, ultrastructural pathology, experimental pathology, histochemistry, immunochemistry and radioisotope procedures will be used.

During the reporting period the following research activities have been in progress: 1) Pathology of Trypanosoma rhodesiense infection in C4-deficient guinea pigs and those with a normal complement system; 2) Pathogenesis of Trypanosoma rhodesiense infection in the dog; 3) Electron microscopic study of central nervous system and renal injuries in cattle experimentally infected with Trypanosoma congolense; 4) Ultrastructural neuropathology induced in mice by Trypanosoma congolense; 5) A study to define the pathogenesis of glomerulonephritis induced in cattle by Sarcocystis cruzi in collaboration with USDA Animal Parasite Institute, Beltsville, MD; 6) Pathology of experimental Leishmania donovani (Khartoum strain) infection in the cynomolgus monkey; 7) Development and evaluation of Mystromys albicaudatus (African white-tailed rat) as an experimental host for New World cutaneous leishmaniasis; 8) Evaluation of a South American isolate of leishmania from Rattus rattus in experimental hosts; 9) Evaluation of rodents as experimental hosts for New World cutaneous leishmaniasis (Leishmania braziliensis; 10) Pathology of chronic Trypanosoma rhodesiense infection in mice (C57BL/6J strain); 11) The pathology of Rickettsia rickettsii in dogs; 12) Comparative histochemistry of Schistosoma; 13) Pathology of IA-4-N-Oxide treated Schistosoma mansoni infected rhesus monkeys; 14) Eticlogy of spontaneous glomerulonephritis in Aotus monkeys; 15) Clinical pathology studies of the Aotus monkey; 16) Effects of increased dietary fiber on zinc and magnesium levels in human volunteers; 17) Investigation of spontaneously occurring thymic necrosis in suckling mice; 18) Histopathology of activity-stress ulcers in rats; 19) TransAmazon epidemiological survey; 20) Wildlife disease surveys; and 21) Pathology in rats with temporary loss of the micturation reflex after destruction of the nucleus of the locus coeruleus.

 Pathology of <u>Trypanosoma rhodesiense</u> Infection in C4-deficient Guinea Pigs and Those With a Normal Complement System.

Recently, Nagle, et al. (1974) described a proliferative glomerulonephritis in rhesus monkeys infected with <u>Trypanosoma rhodesiense</u>. The renal lesion was associated with depression of the serum level of complement and complement component C3 but not C4 and deposition of complement components and immunoglobulins in the mesangium and basement membrane of glomeruli. Both the changes in complement components and immunoglobulins in glomeruli indicated that principally the alternate pathway of complement activation was involved as the effector system in the production of the glomerulonephritis.

To acquire additional information on the role of complement activation by the "alternative pathway" in trypanosomal induced glomerulone-phritis, C4-deficient guinea pig mutants infected with <a href="Trypanosoma">Trypanosoma</a> rhodesiense were employed. Using C4-deficient animals it is possible to see if the glomerulonephritis can develop in the absence of the "classic pathway" of complement activation which requires C4. The study involved light and electron microscopic analysis of the glomerular injury, immunohistochemical localization of complement components and immunoglobulins within the mesangium and glomerular basement membrane, and serum levels of total complement and complement components of C4-deficient mutants and parental normal guinea pigs.

Summary of Results: By light and electron microscopic analysis the onset, nature of injury and severity of the glomerulonephritis were remarkably similar in both C4-deficient and normal guinea pigs. Immunohistochemistry revealed deposits of complement components and immunoglobulins in the glomerular basement membrane and mesangium of glomeruli. C4-deficient animals differed by not having C4 deposits. The serum levels of total complement and complement components were depressed, elevated or remained normal in both infected C4-deficient and normal guinea pigs. No serum C4 was detected in C4-deficient animals.

The study is completed and the results are being assembled for publication.

Pathogenesis of Trypanosoma rhodesiense in Dogs.

This study was conducted to evaluate the gross pathology, histopathology, clinical pathology, immunopathology and electron microscopic pathology of Trypanosoma rhodesiense infection in the dog and its pathogenesis.

Acute studies (less than 21 days) have been completed. The gross pathology, histopathology, clinical pathology and coagulation results in the animals have indicated a disease syndrome occurs similar to that of man and other species infected with <a href="Trypanosoma rhodesiense">Trypanosoma rhodesiense</a>. The findings indicate a generalized systemic disease with the outstanding lesions being a pancarditis, generalized vasculitis, myositis, splenitis, lymphadenitis and a proliferative glomerulonephritis. Those findings and the severe thrombocytopenia suggest the possibility of an active immunologic phenomenon occurring in dogs.

Chronic studies are underway to determine the progression of the lesions and further define the pathogenesis.

 Electron Microscopic Study of Central Nervous System and Renal Injuries in Cattle Experimentally Infected with Trypanosoma congolense.

African trypanosomiasis of domesticated animals is characterized by irregular fever, anemia, emaciation, subcutaneous edema, weakness, sometimes photophobia, and death. Death may follow an acute illness or after a prolonged course during which insiduous body wasting and weakening are the major features. The causative agents in these trypanosomal infections have been <a href="Trypanosoma">Trypanosoma</a> congolense, <a href="T.">T.</a> vivax, and <a href="T.">T.</a> brucei. Trypanosoma <a href="Trypanosoma">Trypanosoma</a> brucei is the type-species for <a href="T.">T.</a> redesiense and <a href="T.">T.</a> vivax, and <a href="T.">T.</a> brucei. Trypanosoma to proceim to trypanosomiasis in man. Recently, it has been determined that <a href="T.">T.</a> rhodesiense occurs in cattle. The biologic behavior of <a href="T.">T.</a> congolense differs from that of <a href="T.">T.</a> rhodesiense in the bovine host and hence the pathogenesis of the lesions induced by this trypanosome may differ. By light microscopy a definite difference can be recognized in the brain lesion between <a href="T.">T.</a> congolense and <a href="T.">T.</a> rhodesiense.

The purpose of this study is to analyze the pathogenesis of  $\underline{T}$ .  $\underline{congolense}$  induced brain and kidney injury and subsequently compare this with the pathogenesis of injury induced by  $\underline{T}$ .  $\underline{r}$  induced by  $\underline{T}$ .  $\underline{r}$  induced by  $\underline{T}$ .  $\underline{r}$  induced by  $\underline{T}$  immunohistochemistry and histochemistry will be utilized during this analysis. This study is approximately 50% completed.

4. Ultrastructural Neuropathology Induced In Mice By Trypanosoma congolense.

In a feasibility study to acquire information on the neuropathology in trypanosomal induced meningoencephalitis, mice were infected with Trypanosoma congolense. Light and electron microscopic techniques were employed to examine the sequential neurologic injury.

It was determined that trypanosomes marginated and adhered to vascular endothelium before damage to these lining cells and leakage of the vascular wall occurred. Margination and adhesion of trypanosomes to the vascular endothelium was observed as early as 45 hours postinfection. It maximizes during high gamma globulin production. Vascular leakage preceded perivascular exudation, cellular infiltration and injury to the adjacent parenchymal tissue.

This study has been completed and preparation of a manuscript has been initiated.

To accurately determine the time interval of blood-brain barrier damage sufficient to allow leakage in  $\underline{T}$ . congolense infected mice a fluorescent dye was injected and brains from infected mice were examined daily with fluorescence microscopy. This study is two-thirds completed.

5. Pathogenesis Of Glomerulonephritis Induced In Cattle By Sarcocystis cruzi.

Bovine calves experimentally infected with <u>Sarcocystis cruzi</u> developed a proliferative glomerulonephritis. The glomerular injury was analyzed by light microscopy, histochemistry, electron microscopy and immunofluorescence microscopy.

It was determined that the causation of the glomerular injury was immune

complexes deposited furing the course of the <u>Sarcocystis</u> infection. The lesion appeared to be transient in animals surviving the acute infection.

This project is completed and manuscript preparations await analysis of serum complement levels.

This investigation consists of pathology support provided to the Animal Parasite Institute (API), USDA, Beltsville, MD. This work and similar investigations were initiated upon request to this department by API.

6. Pathology of Experimental Leishmania donovani (Khartoum strain) Infection In The Cynomolgus Monkey (Macaca fasicularis).

The <u>Leishmania donovani</u> (Khartoum strain)--hamster model is being used in a primary drug screen to test candidate anti-leishmanial drug efficacy. Over a  $2\frac{1}{2}$  year period, 94 of approximately 1170 drugs tested were found to have activity equal to or greater than that of the index drug Glucantime.

Studies were initiated in collaboration with the Division of Medicinal Chemistry and the Department of Parasitic Diseases, WRAIR, to develop a cynomolgus monkey model in which the promising candidate anti-leishmanial drugs could be further tested against leishmanial infections. One previous study indicated that infections with Leishmania donovani (Khartoum strain) could be induced in cynomolgus monkeys with clinical signs and anatomic pathology remarkably similar, in most instances, to the natural disease described from infected human beings. Another study indicated that another commonly used strain of Leishmania donovani, the Hm strain (a Mediterranean strain) was less pathogenic in cynomolgus monkeys than the Khartoum strain and therefore less suitable for the development of a monkey model. A third study was conducted to determine the maximum tolerated dose of the index drug Glucantime that could be used in future efficacy-comparability studies with candidate drugs.

A current study is designed to: 1) verify previously observed clinical signs and anatomic pathology; 2) collect additional baseline data, particularly clinical pathology; 3) determine the efficacy of the index drug Glucantime at different dose levels in curing or suppressing leishmanial infections in cynomolgus monkeys; and 4) determine the toxicity of the index drug at different dose levels.

Preliminary results: All cynomolgus monkeys inoculated with Leishmania donovani (Khartoum strain) developed infections. The infections were accompanied by characteristic clinical signs and changes in hemograms and serum chemistries, although the changes were more variable and, on the average, less severe than expected. Two of the 4 infected, untreated animals died of leishmaniasis. One of the infected, treated animals died 3 days after treatment with 50 mg antimony base of Glucantime per kilogram per day was begun. All of the remaining animals have apparently recovered fully from their infections since parasites are no longer detectable by culture or by microscopic examination of impressions prepared from liver and bone marrow biopsies. The apparent self-cure of the infected, untreated animals may indicate that the cynomolgus is not a good model for studying the therapeutic efficacy of prospective anti-leishmanial compounds. Studies which will evaluate their immune system, especially to determine if they have circulating antibodies against Leishmania donovani prior to the experimental exposure, are needed. Examining the infection in different age

groups may give additional information on their susceptibility to severe leishmanial disease. Since all cynomolgus monkeys inoculated with the Khartoum strain acquired infections, the use of this species as a model for studying prophylactic efficacy of prospective anti-leishmanial compounds appear feasible.

7. The African White-tailed Rat (Mystromys albicaudatus) As An Animal Model For South American Cutaneous Leishmaniasis.

In collaboration with the Department of Biology, WRAIR.

The gross and histopathologic changes of the cutaneous lesions of Leishmania braziliensis panamanensis were observed in Mystromys albicaudatus. This strain of Leishmania, originally isolated from a human being on NNN media, was inoculated into hamsters, reisolated, frozen and maintained at -170°C until used in this study. The promastigotes used in the inoculum were propagated in Schneider's insect media. Necropsies were performed 24, 48 and 72 hours postinoculation, and sequentially every two weeks for twelve weeks. An indurated ulcerative lesion similar to American cutaneous leishmaniasis of man developed in all infected animals. Regional lymphadenopathy was observed one month postinoculation.

Thus, the African white-tailed rat ( $\underline{\text{Mystromys}}$  albicaudatus) becomes the first reliable animal model of New World cutaneous leishmaniasis,  $\underline{\text{L}}$ .  $\underline{\text{b}}$ . panamanensis.

8. Evaluation Of A South American Isolate of <u>Leishmania</u> From <u>Rattus</u> rattus In Laboratory Animal Models.

In collaboration with the Department of Biology, WRAIR.

This isolate of <u>Leishmania</u> was made from a rat in Honduras where the last human death of <u>New World</u> visceral leishmaniasis occurred. This isolate is currently being characterized in laboratory animal models.

 Evaluation of Rodents As Experimental Hosts For New World Cutaneous Leishmaniasis (<u>Leishmania braziliensis</u>).

In collaboration with the Division of Medicinal Chemistry, WRAIR.

Laboratory animals (mice, rats and chinchillas) have been experimentally inoculated with <u>Leishmania braziliensis</u> to determine their value as an animal model to study cutaneous <u>leishmaniasis</u> induced by <u>L. braziliensis</u>.

This experimental work is in progress. The inoculated animals are being evaluated.

10. Pathology Of Chronic <u>Trypanosoma</u> <u>rhodesiense</u> Infection In Mice (C57BL/6J strain).

In collaboration with the Department of Immunology, WRAIR.

The need for a laboratory rodent model to study chronic <u>Trypanosoma</u> rhodesiense infection is well-recognized and well-publicized. Recently, it was determined by investigators in the Department of Immunology, WRAIR, that

C57BL/6J strain mice developed chronic trypanosomiasis when exposed to  $\underline{\mathbf{T}}$ . rhodesiense.

The current study employing light and electron microscopy, immuno-fluorescence microscopy, histochemistry and immunohistochemistry will evaluate the pathogenesis of this chronic infection in mice. This study is approximately 50% completed.

### 11. The Pathology Of Rickettsia rickettsii In Dogs.

Dogs infected with Rickettsia rickettsii developed a generalized infection characterized by fever, malaise, prostration, skin lesions and enlargement of the spleen and liver. Microscopically, endothelial cells of small blood vessels were swollen, necrotic and accompanied thrombosis of the vessels. Vascular lesions were prominent in the skin, but vasculitis occurred in many organs. In the brain aggregations of lymphocytes, polymorphonuclear leukocytes, and macrophages were associated with blood vessels of the gray matter. The small blood vessels of the heart were similarly involved.

This study is completed and a manuscript is being prepared for  $\operatorname{publication}$ .

### 12. Comparative Histochemistry Of Schistosoma.

This study has been completed after the examination of eight species of schistosomes in one or more of six host species. In addition to H&E evaluation, three acid-fast staining methodologies were evaluated. Only S. mansoni was uniformly positive and then the lateral spine was the most intensely stained portion. S. japonicum and S. intercalatum were not positive in our study in contrast to other reports in the literature. Our conclusion is that while the acid-fast stain may be of confirmatory use in some cases, it is not a useful method for routine screening and precise diagnosis of field cases.

### 13. Pathology Of IA-4-N-Oxide Treated S. mansoni Infected Rhesus Monkeys.

Phase I is completed and was published in the September 1977 issue of  $\frac{\text{Trop. Med.}}{\text{Department}}$ . This collaborative study is being conducted with the Department of Parasitic Diseases, DCD&I, and the Department of Biology, Division of Medicinal Chemistry, WRAIR. Phase I documented the effects of IA-4-N-Oxide, an Hycanthone analog, administered orally vs intramuscularly and prophylactically vs therapeutically. Therapeutic treatment by either route is highly effective.

Phase II is also completed and a manuscript is in preparation. This study compared the efficacy of IA-4-N-Oxide with IA-3-N-Oxide, another analog of Hycanthone, which is cheaper and can be produced in greater quantity. Results indicate that IA-3-N-Oxide is equally as effective when given therapeutically either orally or intramuscularly.

Phase III is in progress and is designed to test the efficacy of IA-4-N-Oxide against the Lowell strain of  $\underline{S}$ .  $\underline{mansoni}$  since at present there is conflicting data available concerning the efficacy of this drug. This

project is being conducted concurrently with one at Lowell University using the WRAIR strain of S. mansoni in Cebus apella, the Lowell host system.

14. Etiology Of Spontaneous Glomerulonephritis In Aotus Monkeys.

Gross and histopathologic examination of Aotus monkeys dying spontaneously in the WRAIR colony have revealed a high incidence of severe proliferative glomerulonephritis. Further analysis of the glomerulonephritis indicate that it is of immune complex origin. In collaboration with the Department of Animal Resources and the Department of Immunology, WRAIR, efforts are currently in progress to determine the etiology of this immune complex disease.

15. Clinical Pathology Studies Of The Aotus Monkey.

In collaboration with the Division of Veterinary Resources, a project is underway to define the hematological and serum chemistry characteristics of the Aotus monkey. This data is currently unavailable and will form the basis for making clinical interpretations of the WRAIR Aotus colony and will assist investigators involved in the study of glomerulonephritis and gram negative sepsis in this colony.

To date some 300 samples have been analyzed and the data is being evaluated. In addition to routine hematology and clinical chemistry analyses, serum electrophoretic studies are ongoing and hemoglobulin electrophoretic studies are in the pilot stage.

16. Effects Of Increased Dietary Fiber On Zinc And Magnesium Levels In Human Volunteers.

In collaboration with the Food and Drug Administration and the Armed Forces Institute of Pathology, a study was conducted to determine the effects of increased dietary fiber on zinc and magnesium levels. Additional hematological and clinical chemistry parameters were monitored during the study to protect the interests of the volunteers and maximize the knowledge gained concerning the effects of fiber intake.

The study is complete and the data is being analyzed at present.

17. Investigation Of Spontaneously Occurring Thymic Necrosis In Suckling Mice.

This study was initiated in an effort to determine the etiologic agent(s) responsible for a natural outbreak of thymic necrosis in 3 to 6 day old mice from the WRAIR mouse production unit. Thirty-six litters having spontaneous deaths within 24 hours were screened histopathologically and tissues were saved for electron microscopic and immunofluorescent antibody studies. Test procedures are currently being implemented to detect the presence of a suspected viral infection.

18. Histopathology Of Activity-Stress Ulcers In Rats.

This study has been completed and a manuscript titled, "A Microscopic Examination of the Activity-Stress Ulcer in the Rat", has been submitted to the Journal of Physiology and Behavior for publication.

# 19. TransAmazon Epidermiological Survey.

To date 600 rodents, marsupials, bats and other miscellaneous mammals have been examined histologically. The report of histological lesions has been furnished the WRAIR Team-Belem, with a summary of etiologic agents observed and comments on future studies. This completes Phase I.

Specific plans are being formulated to examine another group of specimens and to selectively collect specimens of Proechimys sp. This particular rodent has a high prevalence of pulmonary adenomatosis (minimum prevalence - 23%) and a corresponding rate of infection with an unidentified larval nematode (minimum prevalence - 28%). Many of the hyperplastic/neoplastic nodules have one or more sections of larval nematode within the lesion. Of 99 lungs observed 15 (15%) had both adenomatosis and larval nematode in the same section. Had multiple or step section been prepared of blocks on hand, the prevalence of adenomatosis and correlation of nodules and parasites would undoubtedly be higher. It will be important to document this relationship since few parasites are known carcinogenic agents and can be utilized as research models.

### 20. Wildlife Disease Surveys.

Collaborative support is being provided for projects based at Ft. Stewart, GA and Ft. Bragg, NC. Two hundred thirty-seven animals were surveyed during the 1976-77 hunting season and the tissues are being evaluated. Correlation of histology and serology data is underway in an attempt to compile a comprehenvise picture of the zoonotic disease trends at Ft. Stewart and Ft. Bragg.

21. Pathology In Rats With Temporary Loss Of The Micturation Reflex After Destruction Of Areas In The Region Of The Nucleus Of The Locus Coeruleus.

Gross and microscopic pathology support is being provided the Department of Microwave Research in their studies of the various behavioral and physiologic changes occurring in rats after destruction of various specific areas in the region of the nucleus of the locus coeruleus. The support is currently limited to those animals demonstrating temporary loss of the micturation reflex. Clinical changes seen are distended abdomens, urine-soaked peri-anal and posterior abdominal areas, hematuria, hemorrhagic anogenital organs, and behavioral changes characterized by biting and manipulating the anogenital area in apparent attempts to expel urine from the urinary bladders. Gross pathologic changes seen are distended urinary bladders containing bloody urine and hemorrhagic penises and penile sheaths. To date, tissues from eleven affected animals have been collected, processed and are being evaluated.

RESEARCH	AND TECHNOLOG	Y WORK UNIT S	UMMARY			2. DATE OF SU			CONTROL SYMBOL
1 DATE PREV SUM'RY	4. KIND OF SUMMARY	S. SUMMARY SCTY	S. WORK SECURITY	DA OF	6530	77 10	OL SPECIFIC		S. LEVEL OF SUM
76 10 01	D. Change	U	U	NA		NL	CONTRACTO	ACCESS	A WORK UNIT
10. NO./CODES:*	PROGRAM ELEMENT	PROJECT	NUMBER	TASK ARE	A NUMBER		WORK UNI		
- PRIMARY	61102A	3M16110	2BS01		0	138			
b. CONTRIBUTING									
XXXXXXXXXXXX	Cards 114F								
	Security Classification Code								
(U) Vac	cine Developm	ment in Try	panosomias:	ıs					
		O Migrobio	logr						
13. START DATE	Biology 01010	14. ESTIMATED COM	PLETION DATE	15. FUNDIN	AGENCY		16. PERFOR	ANCE MET	тнор
73 09		CONT		DA	1	1	c. :	In-Hou	ise
17. CONTRACT/GRANT				18. RESOU	CES ESTIMAT	E & PROFES	SIONAL MAN YE	_	DS (In thousands)
& DATES/EFFECTIVE:	NA	EXPIRATION:		100	ECEDING		_		75
b. NUMBER:*				FISCAL	77		3		75
C TYPE:		& AMOUNT:		YEAR EL	78		3		85
& KIND OF AWARD:		f. CUM. AMT.					3		0.5
19. RESPONSIBLE DOD	DRGANIZATION			PERFOR	MING ORGANI	ZATION			
MAME: Walte	r Reed Army	Institute o	f Research	HAME:	S Army	Medical	Resear	ch Uni	t-Kenya
				ADDRESS.*	Kal	oete, Ke	nva		
ADDRESS:* Wa	shington, D.C	20012		AUDINESS!		,			
				PRINCIPAL	INVESTIGATO	R (Fumieh SEAN			
RESPONSIBLE INDIVIDU				HAME.*					
	pmund, Garris	son, COL		TELEPHO		vatch, R	obert M	., LTC	: VC
	2-576-3551								
21. GENERAL USE				4	INVESTIGATO				
				HAME:	Mu	riithi,	I. Dr.		
	reign intelli		considered	NAME:	We:	llde, Br	uce T.		
22. KEYWORDS (Procede	BACH with Security Classifi	cetton Code)							
	; (U) Trypano								
	IVE. 24 APPROACH, 25.								
	e objective o								
	rican trypano								
	fits include			_		-			-
	onse and pathould a								
	d constitute								
endemic ar		a scrious	nazara zor		L' Poli	Joinier o	peracri	,	
	periments cor	ducted at	WRATE and	in Kens	a have	demonst	rated th	nat ex	merimenta
	in be successi								-
	s can be rend								
	se. Partial i		_					01011	
	07 - 77 09 0							a. sho	w that
	nd 21 of 29 i								
	ra, respectiv				-	-			
	iserum. This								
	this period			-				-	Complete
	to T. congol								•
	ppears to be				_				
	s complementa			-	-		-	-	
	iasis.for tec	•							
	eport 1 July	and the same of the same of							
-									
				05					

PROJECT 3M161102BS01 RESEARCH ON MILITARY DISEASES

Work Unit 138 Vaccine Development in Trypanosomiasis

Investigators.

R. M. Kovatch, LTC, VC

B. T. Wellde, DAC

W. T. Hockmeyer, MAJ, MSC

### SECTION A

ANTIGENIC RELATIONSHIPS BETWEEN ORGANISMS OF THE TRYPANOSOMA BRUCEI SUBGROUP IN THE LAMBWE VALLEY, SOUTH NYANZA, KENYA

PROGRAM AND BACKGROUND: To determine the extent of antigenic variability in trypanosomes of the  $\underline{\mathtt{T}}.$   $\underline{\mathtt{brucei}}$  group collected from man and animals in the Lambwe Valley. T. rhodesiense is endemic in the Valley causing periodic disease in man. Domestic and game animals harbor the morphologically indistinguishable <u>T. brucei</u>. This project is designed to study reactions of various antisera to determine the variability of different antigenic types within the parasitic population. The findings will, in part, determine whether or not immunization could be a practical means of controlling the disease. Trypanosomes collected from adjacent countries will also be studied to determine the geographic extent of similar antigenic types. The antigenic relationship between the parasites of man and animals of these areas will also be examined. Immunization against African trypanosomiasis appears to be dependent in large part on the number of antigenic types of the parasite found in a given area. Gray (1970) examined the same herd of cattle for five years in Nigeria and reported the presence of numerous different antigenic types of T. brucei. However, while working with T. gambiense isolated from different endemic areas in Nigeria, he found similarity of basic antigenic types (Gray 1972, 1975). In Lambwe Valley human rhodesian sleeping sickness is endemic and domestic and game animals harbor T. brucei. Since the trypanosomes of man and animals are morphologically indistinguishable, their relationship to each other remains questionable. In hearby Alego Station T. brucei-like organisms were isolated from cattle and transmitted to human volunteers. These people developed typical T. rhodesiense-like infections (Onyango 1966).

PROGRESS: Isolates of the <u>T. rhodesiense</u> parasites were collected from patients at the Homa Bay Hospital on Lake Victoria, western Kenya, by members of the Kenya Medical Department. Blood was injected IP into rats which were then transported to us for study. Two strains of <u>T. rhodesiense</u> from Gambella, Ethiopia, were collected by the US Navy Research Unit, Addis Ababa, Ethiopia. Isolates of trypanosomes were tested by

neutralization (Soltys 1957) with antiserum collected from bovines which had undergone long-term infections with various isolates. To date, 46 isolates of T. rhodesiense have been acquired from trypanosomiasis patients in the valley. The dates of isolation range from 1970 until the present time. Two antisera have been used to assay these isolates: one from an animal infected with an isolate made in 1972 and the other from an animal infected with a strain obtained in 1974. The duration of infection in the two serum donor animals was 227 and 279 days, respectively. Twenty two of thirty five isolates (62.8%) tested were neutralized by the first antiserum while the second antiserum neutralized 23 of 31 isolated (74.2%) tested thus far. Eighteen isolates were neutralized by both antisera while 5 isolates did not react with either antiserum. T. rhodesiense isolates from Ethiopia and two different areas in Kenya do not appear to react with either antiserum prepared against Lambwe Valley isolates.

During the past year a number of cases of trypanosomiasis were reported from an area in Kenya near the Uganda border. We obtained three isolates from humans and 8  $\underline{\text{T.}}$   $\underline{\text{brucei}}$  isolates from cattle in the same area. An antiserum prepared against one of the human isolates neutralized all three isolates from humans. When tested against four of the  $\underline{\text{T.}}$   $\underline{\text{brucei}}$  isolates from cattle, the antiserum neutralized all of them.

When isolates of  $\underline{\mathbf{T}}$ . brucei from cattle in Lambwe Valley were tested, the antiserum showed a strong effect on 5 of 25 isolates. No parasites appeared in mice given trypanosomes incubated with immune serum at any dilution. Two other isolates were neutralized at  $10^3$  and below; however, mice at  $10^4$  were positive. The nature of this partial reaction is unknown but these isolates will be retested. It is reasonable to assume that these reacting parasites from cattle are  $\underline{\mathbf{T}}$ . Thodesiense since the neutralization test is variant specific. The percentage of cattle parasites which react with the antibody is surprising and indicates that cattle may be a more important aspect in the cycle of the disease than previously thought.

### SECTION B

### STUDIES ON TRYPANOSOMA RHODESIENSE IN CATTLE

PROGRAM AND BACKGROUND. During our initial immunological studies of the irradiated vaccine and antiserum production in cattle, we noted that some animals underwent a severe form of disease. In general  $\underline{\mathbf{T}}$ .  $\underline{\mathbf{T}}$  rhodesiense has been reported as being non-pathogenic for cattle. We have, therefore, carried out additional experiments to confirm our original findings and to compare the disease process in bovines with that which occurs in man.

PROGRESS. Ten animals in our study developed disease characterized by weight loss, fever, pleocytosis and CNS disorders. Uncoordinated movements, circling and opisthotonus were observed (Table 1B). Fever and leucopenia were common during the onset of patent parasitemia and were followed by a leucocytosis. Terminal WBC levels were somewhat reduced (Table 2B). Generally, there was an increase in packed cell volumes early in the disease although mild to moderate anemia developed as the infection progressed. Cerebrospinal fluid from infected animals had increased levels of leucocytes made up primarily of lymphocytes. Total protein levels of cerebrospinal fluid were also increased. Gamma globulin was detected by electrophoresis and spinal fluid was positive for complement fixing antibody.

Five additional bovines were autopsied during the period of this report. Gross observations as documented in our previous progress report included thickened dull grey meninges over the dorsal aspects of the brain and prominent lymph and hemolymphadenopathy. Histologically severe meningoencephalitis remains the salient histological feature. The results are summarized in Table 3B. The most severe changes are noted in the white matter of the central nervous system where mixed perivascular plasmacytic and lymphocytic infiltrates and focal and diffuse gliosis are important features. Demyelination is almost exclusively limited to perivascular areas. Although involvement of the spinal and peripheral nerves is minimal, limited infiltrates of inflammatory cells in the neural sheath and perivascular spaces can be found on thorough search. Of interest, in three animals autopsied between 84 and 108 days post inoculation a moderate to severe pancarditis was found. Myocytolysis and sarcolemmal cell hyperplasia accompanied by infiltrates of macrophages, lymphocytes and plasma cells were evident. The epicardium and endocardium also had infiltrates of inflammatory cells. The lungs of these animals had large numbers of hemosiderin laden macrophages in the alveolar walls. Pulmonary hemosiderosis is frequently associated with cardiac insufficiency.

The presence of severe meningoencephalitis with the most extensive lesions in the white matter of the central nervous system in our material is compatable with the leukoencephalitis observed in humans with chronic trypanosomiasis (Caldwell, 1937; Manuelidis, 1967). The findings of myocardial lesions in our animals that died after a relatively short clinical course is similar with the cardiac syndrome associated with acute <u>T. rhodesiense</u> infections of man (Ormerod, 1970).

Detection of parasites in <u>T. rhodesiense</u> infected cattle is difficult after the fourth month of infection if one relies on sub-inoculation of blood into rats. Small quantities of lymph node aspirate (usually less than 0.1cc expanded in 1.0cc of 10% fetal calf serum) injected into rats appears to be a more effective method if isolating the parasite late in the course of infection. Examination of Giemsastained lymph node smears is also less efficient than lymph node aspirate subinoculation. The results between 15-30 months post inoculation are included in Table 4B. The lymph node aspirate technique may be a useful adjunct in the detection of infections in the field and the detection of infection in animals to be used in future immunization studies. The detection of parasites in lymph node aspirates of our animals for lengthy periods is not unlike the persistence of infection in chronic human trypanosomiasis.

PATHOGENIC INFECTIONS OF TRYPANOSOMA RHODESIENSE IN CATTLE

			*				*				
* SES	CSF	N.D.	Neg.	N.D.	Neg.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.
DETECTABLE PARASITES	Lymph node	N.D.	Pos.	N.D.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.
DETE	Blood	Neg.	Pos.	Neg.	Neg.	Pos.	Neg.	Neg.	Pos.	Pos.	Pos.
Duration of	Disease (Days)	179	227	582	714	279	703	301	108	92	84
Clinical	signs	1,3,5,6	1,6	1,3,4,6	1,4,6	1,4,6	1,3,4,6	1,4,6	1,6	1,6	1,6
Infecting	strain	Wellcome	LVH-1	LVH-1	LVH-2	6-нл	LVH-12	LVH-12	LVH-28	LVH-29	LVH-29
	An. No.	268	6882	243	7304	7307	8601	7859	16	8888	8901
	A	1.	2.	3.	4.	5.	.9	7.	8	.6	10.

At necropsy by subinoculation into mice

\*\* Clinical signs:

1. Unco-ordinated movements 4. Hypersensitivity
2. Circling 5. Opisthotonos
3. Tremor 6. Weight loss

\*\*\* Spinal cord positive

LEUCOCYTES IN TRYPANOSOMA RHODESIENSE INFECTED CATTLE (x1000)

ANIMAL NUMBER	PRE INFECTION	SECOND WEEK	HIGHEST VALUE (AND DAY)	TERMINAL VALUE (AND DAY)
16	11.9	16.3*	17.9 (25)	15.1 (108)
243	9.6	7.7	34.3 (51)	18.7 (551)
268	14.6	12.6	18.6 (36)	10.8 (179)
6882	11.2	9.1	18.8 (26)	9.4 (227)
7304	12.6	7.9	20.8 (558)	20.7 (714)
7307	15.4	23.4	25.8 (14)	15.6 (279)
8601	14.6	9.3	37.2 (586)	18.2 (703)
8888	16.7	19.5	19.9 (25)	16.1 (91)
8901	10.5	12.9*	21.9 (58)	15.5 (84)

<sup>\*</sup> THIRD WEEK

TABLE 3B

OGICAL CHANGES IN THE CNS OF CATTLE INFECTED WITH TRYPANOSOMA RHODESIENSE

1,3,5 1,3-8 1,3-8,1c 1,3-5,7,8 1,3-5,7,8 1,3-8 1,3,5-8 1,3,5-8 1,3,5-8 1,3,5-8 1,3 1,3 1,3 1,3 1,3 1,3 1,3 1,3	MIDBRAIN CORTEX- BASAL MIDCORTEX CORTEX- PLTUITARY SEE OCCIPITAL LB. GANGLIA OLFACTORY COMMENT	1,3,5,6 1,3-5,8 1,3-6,8 1,3,5,8,9 1,3,5,8 1,3,7 A	1,3-5,7,9 1-9,11 1-11 1-9,11,12 1-5,7,8 3,4,8,10 B	1,3,5,7 N/A 1-8,10,12 1-3,5,7,8 N/A 3,4,8,13 C	3,7 1,3 3 N/A N/A 3,8,13 D	0 1,3-5,7 1-10 1-3,5-8, 1-5,7-9,11 1-9 3,4,8,13 E	1,3,6,7,11 1,3-5,8 1,3 1-5,8,9,11 1,3-5,8 3,13 F	1-8 1-10 1-11 1-3,5-10,12 1-10 1,3,7 G	1,3-5,7,8, 1-5,7-9,15 1,3-9,15 1-11,15 1-9,15 1,3,4,8 H	1,3,5 1,3-5 1,3,5 1,3,5 1,3-5 1,3,7 I	1,3 N/A N/L N/A 1 1,3,7 J	sections not identified
1,3,5 1,3,5 1,  1,3,4,7 1,3-8 1,  N/A 1,3-5,7,8 1,  1,3,4 1,3-5,8,10 1,  1,3,5,7 1,3,5-8 1,  1,3,5,7 1,3,5-8 1,  1,3,5,7 1,3,5-8 1,  N/A 1,3-8 1,  NOt available or tissue sections Weningeal infiltrations Subplat gliosis Perivascular infiltrates Woott's cells Vasculitis												
CERVICAL SPINAL O 1,3,5 1,3,4,7 N/A 1,3,4 1,3,5,7 1,4,7 1,4,7 1,4,7 1,5,	CERVICAL CEREBELLUM MII SPINAL CD.						5,7,8	1,3,5-8				Not available or tissue sectio No lesions Meningeal infiltrations Subpial gliosis Perivascular infiltrates Mott's cells Vasculitis

### ADDITIONAL COMMENTS FROM TABLE 3B

- A. The salient feature in the brain of this case was a lymphoplasmacytic infiltrate in the meninges and perivascularily at all levels of the CNS examined. Only a few Mott's cells were found. Gliosis was irregularly diffuse in the subcortical white matter with gemistocytic astrocytes limited to a single section. The pituitary had a marked glial-inflammatory infiltrate limited to the neurohypophysis. Additional changes included marked serous atrophy and chronic inflammation of fat in the coronary groove of the heart. A mixed infiltrate of lymphocytes and plasma cells were noted to be most intense in the myocardial interstitium adjacent to the epi and endocardium. All lymphoid organs were reactive with follicular hyperplasia and marked accumulation of lymphocytes and plasma cells in the medullary sinusoids.
- B. Lesions were most severe in the subcortical white matter but extensive lesions were present in the grey matter as well. Marked cystic cavitations primarily surrounding blood vessels near the external capsule of the basal ganglia, extensive astrocytosis of the white matter and exaggerated sulci indicates marked cerebral atrophy. The inflammatory reaction was mainly plasmacytic and Mott's cells were numerous.
- C. Lesions in the brain sections examined were most severe in the white matter of midbrain and basal ganglia. The inflammatory reaction was primarily lymphocytic. Mott's cells were infrequent. The stroma of the chorid plexus of the 4th ventricle was edematous and the perivascular areas were infiltrated with lymphocytes.
- D. The least severe histological changes were found in this case. The inflammatory reaction in the CNS was primarily lymphocytic.
- E. The inflammatory reaction was mixed plasma-lymphocytic with a few neutrophils, and most severe in myelinated areas of the brain. There was extensive subpendymal gliosis, malacia and vacuolation near the lateral ventricles.
- F. The inflammatory reaction was primarily lymphocytic and much more extensive in the myelinated portions of the CNS than in the grey matter. Peripheral nerve involvement was noted in the optic, trigeminal, sciatic and brachial nerves. It consisted of a mild perivascular and neural sheath infiltrate.
- G. Lesions were most severe in the subcortical white matter, basal ganglia and white matter of the cerebellum. Salient features included marked perivascular infiltrate, composed of lymphocytes, plasma cells, histiocytes and irregular diffuse gliosis. Gemistocytic astrocytes were numerous. Occasional gitter cells were found in areas most severely involved. Large lakes of proteinaceous fluid surrounded several arteries. The gliosis in the cortex was most frequently focal. Mott's cells in this case were not numerous. Also in areas of intense inflammation reaction, degeneration and glial satellitosis of neurons were occasionally found. Marked reactive hyperplasia of all lymphoid organs and mild pancarditis were other important histological alterations.

- H. The inflammatory reaction in the brain was mixed plasma lymphocytic with a severe reaction at all levels of the CNS examined. Mott's cells were numerous. The most severe reactions were found in the subcortical white matter where the gliosis appeared generally diffuse and perivascular infiltrates extensive. In the cortex focal glial infiltrates were found. In the spinal cord the inflammatory reaction was most severe in the distal lumbar and sacral portions. Spinal nerve involvement was limited to a mild pial and perivascular infiltrates. Of the peripheral nerves examined, only the brachial had a single small perivascular infiltrate.
- I. The inflammatory reaction in the brain was limited to a moderately severe meningeal and perivascular infiltrate of lymphocytes and plasma cells. Few Mott's cells were found. The most interesting change was a severe pancarditis. Extensive interstitial infiltrates in the myocardium appeared especially intense surrounding Purkinje fibers. Myofibril degeneration with marked sarcolemmal cell proliferation and the presence of large numbers of cardiac histiocytes indicates a reaction of long standing. Chronic steatitis is evidenced by an infiltrate of lymphocytes and plasma cells and clusters of reticuloendothial cells. The alveolar walls of the lung were thickened and contained abundant iron positive brown pigment. Reactive hyperplasia was prominent in all lymphoid organs examined.
- J. The lesions in the brain in this case were minimal with the meninges and perivascular spaces in the brain containing a mild multifocal infiltrate of plasma cells and lymphocytes. The heart was extensively damaged and the lesions characterised by focal myofibril degeneration with accumulation of engorged macrophages and proliferated sarcolemmal cells. Infiltrates of plasma cells and lymphocytes were also noted multifocally in the mycardium as well as the epi and endocardium. The lung was congested with alveolar walls thickened. Macrophages in the walls contained abundant iron positive pigment.

TABLE 4B

RELATIVE EFFECTIVENESS OF DIRECT LYMPH NODE SMEAR EXAMINATION COMPARED TO IP INOCULATIONS USING BLOOD AND LYMPH NODE ASPIRATES FOR DETECTION OF CATTLE HARBORING TRYPANOSOMA RHODESIENSE

# DURATION OF INFECTION IN MONTHS

																		1
		15	15 16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	
BLOOD (5ml)	*н	6/0	6/0	6/0	6/0	6/0	6/0	6/0	6/0	8/0	8/0	8/0	8/0	8/0	8/0	8/0	8/0	
ir 10 kais	* v	c* 0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	
LYMPH NODE	I	4/9	4/9	3/9	3/9	2/9	3/9	3/9	3/9	1/8	1/8	1/8	1/8	1/8	1/8	1/8	1/8	
ASFIRATE IF TO RATS/MICE	U	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	
LYMPH NODE	н	4/9	2/9	2/9	2/9	1/9	2/9	1/9	1/9	1/8	8/0	1/8	1/8	1/8	1/8	1/8	1/8	
SMEAKS	U	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	
The second secon																		١

\* INFECTED ANIMAL

\*\* CONTROL

### SECTION C

### ANEMIA IN TRYPANOSOMA CONGOLENSE INFECTED CATTLE

A total of 22 experimental and 13 control cattle were used to study the anemia caused by T. congolense infections. Early in the course of infection decreases in packed cell volume, erythrocyte concentration and hemoglobin occurred and coincided with increases in both mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH). The indices reached the highest levels during the period between eight and twelve weeks after infection and were accompanied by a reticulocyte (Ret.) response. By week 20 these values (MCV, MCH, Ret.) had decreased to pre-infection levels even though the anemia persisted (Table 1c). The apparent half life of chromium-51 labelled erythrocytes in infected animals was approximately 40% of that of controls at eight weeks. In surviving animals the half life gradually returned to normal as the parasitemia level decreased (Table 2c). Chromium was excreted in the urine of infected animals at higher levels than that of controls indicating an excessive destruction of labelled erythrocytes and excretion of chromium by the kidney (Table 3c). No differences in chromium levels in fecal samples were found between experimental and control animals (Table 4c). Total blood volumes of infected and control animals did not differ significantly throughout the course of infection (Table 5c). Plasma volumes of infected animals increased and these animals underwent a corresponding decrease in erythrocyte volume (Tables 6c and 7c). Serum iron levels which were elevated at eight weeks post infection decreased to low levels by week 28. In surviving animals or after treatment, the serum iron levels returned to normal. Early during the infection the plasma iron turnover (PIT) measured with iron-59 was greater than that of controls indicating increased erythrocyte production; however, by week 28 the PIT was only 38% of that occurring in controls indicating the presence of a severe dyshemopoiesis at this time. Iron-59 incorporation followed a similar pattern. Whereas 50% of the injected dose of iron reappeared in controls in an average of 6.9 days, it took only 4.9 days to appear in animals at eight weeks. Later, however (28 weeks), eight days were required before 50% of the injected dose of iron reappeared in erythrocytes in infected animals. In surviving and treated animals PIT's returned to normal. Thrombocytopenia was found to be a prominent feature of T. congolense infections in cattle and appears to be inversely related to the level of trypanosomes in the peripheral circulation. Chronically infected animals with low levels of parasites had less severe thrombocytopenia and thrombocytes were usually found in normal or elevated numbers when parasites could not be observed. Decreased survival time of chromium-51 labelled thrombocytes was found in infected animals. Chemotherapeutic cure of animals with thrombocytopenia resulted in a rapid elevation of thrombocyte levels to higher than normal values. Leucocyte concentrations followed a similar but less marked course. Preliminary coagulation studies indicated that partial thromboplastin times were

extended after six weeks of infection and that protamine sulfate tests became positive. Fibrinogen levels were lower but no change was noted in prothrombin times. It appeared that the spleen did not contribute greatly to the breakdown of erythrocytes since splenectomized calves developed anemia at a similar rate to that of non-splenectomized controls.

FACKED CELL VOLUMES IN T. CONGOLENSE
INFECTED CALVES \$\frac{t}{2}\$ 25.E.

		II	nfected				Cont	rol		
Weeks										
Wee	7	8	8B	10	Mean	5	6	3	9	Mean
0	34	36	34	34.5	34.6+0.9	29	35	31	33	32.0-2.6
1	30	34	28.5	31	30.9 <sup>±</sup> 2.3	29	35	29.5	31	31.1-2.7
2	26	29	26	30	27.7 + 2.1	32	33.5	33.5	31	32.5-1.2
3	20	27	23.5	27.5	24.5-3.5	30	29	29	33	30.2-1.9
4	23	25	22	30.5	25.1-3.8	33	30	32.8	33.3	32.3 <sup>±</sup> 1.5
5	22	21	24.5	29.5	24.2-3.8	29	27	33.5	37.5	31.7-4.7
6	22	25	18.5	24.5	22.5-3.0	30	27	32	34.5	30.9-3.2
7	23	20.5	19.5	25	22.0-2.5	32	31	32	35	32.5-1.7
8	24	21	17.5	22.5	21.2-2.8	35	38	31	33	34.2-3.0
9	22	22	18.5	22.5	21.2-1.8	33	32	30	34	32.2-1.7
10	24	21	18.0	21.5	21.1-2.5	34	34	30	34.5	33.1-2.1
11	24	23	15.5	20	20.6-3.8	34	38	30	34	34.0-3.3
12	24	21	19.0	23.5	21.9 - 2.3	30	33	33.5	33	32.4-1.6
13	24	22	22	24.5	23.1-1.3	32	35	33	31.5	32.9+1.5
14	24	22	24	26.5	24.1-1.8	32	35	33	31.5	32.9 <sup>±</sup> 1.5
15	26	22	21.5	27.5	24.2-3.5	32	36	34.5	31.5	33.5-2.1
16	26	22	21	27.5	24.1-3.1	32	35	34.5	31	33.1-1.9
17	27	20	18.5	24	22.4-3.9	32	34	30	28	31.0-2.6
18	27	19	18	25	22.2-4.4	33	36	31	30	32.5-2.6
19	25	19	22.5	29.5	24.0-4.4	31	33	36.5	34.5	33.7-2.3
20	25	23	_	-	24.0-2.0	30	31		-	30.5-1.0
21	27	26	22	28	25.7-2.6	28	31	36.5	38	33.4-4.7
22	25	22	-	-	23.5-3.0	28	30	-	-	29.0-2.0
23	27	23	_	-	25.0-4.0	29	29		-	29.0
24	28	27	_	-	27.5-1.0	32	31	-	-	31.5-1.0
25	27	25	-	-	26.0-2.0	28	28	_	-	28.0
26	27	23	_	-	25.0-4.0	31	30	-	-	30.5-1.0
27	-	_	22	28	25.0+6.0	-	-	36.5	38.0	37.7 <sup>±</sup> 1.2
28	28	27	_	-	27.5 <sup>±</sup> 1.0	33	35	-	-	34.0
29	26	23	-	-	24.5 <sup>+</sup> 3.0	28	28	-	-	28.0 <sup>±</sup> -

TABLE 2c

51 Cr ERYTHROCYTE SURVIVAL (Th) +25.E.

Weeks	<b>4-6</b> 6-8 10-12 12-14 18-20 22-24 28-30	31 322 348 360 355 323 299	83 310 334 341 314 338 310	17 339 318 347	55 381 360 332	321.5±30.1 338.0±31.0 340.0±18.1 345.0±11.7 344.5±41.0 330.5±15.0 304.5±11.0	34 172 192 165 288 290 265	20 121 105 125 151 194 222	72 102 87 108	85 147 139 145	203.5-49.9 127.8-46.5 135.5-30.5 130.8-46.2 135.8-24.7 219.5-137.0 242-96.0 243.5-43.0
	12-14	360	341	347	332	345.0-11.7	165	125	108	145	135.8-24.7
Weeks	10-12	348	334	318	360	340.0+18.1	192	105	87	139	130.8-46.2
	8-9	322	310	339	381	338.0-31.0	172	121	102	147	135.5-30.5
	4-6	331	283	317	355	321.5 <sup>+</sup> 30.1	134	120	72	185	127.8-46.5
	2-4	346	336	312	376	342.5 <sup>+</sup> 26.5	166		160	265	203.5-49.9
	0-5	324	319	326	319	322 <sup>+</sup> 3.6	286	260	246	301	273-24.8
đn	019		70:	ntuo;	)			cred	lnfe		
9	No.	5	9	3	6	Mean	7	ω	8B	10	Mean

819

TABLE 3c

# 51 Cr URINARY EXCRETION RBC EQUIVALENTS (ml.)

ha Na	Crown		Weeks	
An. No.	Group	2-4	6-8	12-14
5	Control	40.4	32.8	46.0
6	п	39.1	34.5	43.8
3	п	36.1	46.8	53.6
9	"	42.5	58.0	46.1
Mean		39.5 <sup>+</sup> 2.7	43.0-11.8	47.4+4.3
7	Infected	69.5	117.6	391
8	"	83.8	288.0	163.9
8B	"	82.3	383.0	168.1
10	"	75.2	120.1	139.4
Mean		77.7 <sup>+</sup> 6.6	227.2-131.0	215.6+117.6

TABLE 4c

51<sub>Cr</sub> FECAL EXCRETION RBC EQUIVALENT (ml.)

An. No.	Group		Weeks	
		0-4	4-8	10-14
5	Control	1.7	2.5	2.4
6	п	2.2	2.7	2.6
3	п	2.8	2.8	3.0
9	"	1.4	1.6	1.9
Mean		2.03+0.6	2.4-0.4	2.6+0.5
7	Infected	2.4	2.1	1.9
8	"	2.0	3.4	2.6
8в	"	2.3	2.3	2.2
10	u u	2.7	2.7	1.9
Mean		2.4-0.3	2.6+0.6	2.2-0.3

TABLE 6c

PLASMA VOLUME ML./KG. (+ 2S.E.)

			r	Weeks		
An. No.	Group	0	4	10	18	28
5	Control	31.2	33.6	31.1	33.7	33.6
6	ıı ı	31.6	39.0	35.0	35.3	32.2
3	u	28.6	30.0	32.5	31.6	_
9	"	27.8	30.9	32.5	29.9	-
Mean		29.8 -1.9	33.4 <sup>+</sup> 4.1	32.8 <sup>±</sup> 1.6	32.6 <sup>+</sup> 2.4	32.9 -1.4
7	Infected	33.0	44.6	40.5	37.5	38.3
8		28.3	38.4	39.3	42.8	36.7
8в		26.5	36.0	42.8	37.0	-
10	"	33.3	32.5	35.0	35.4	-
Mean		30.3 ±3.4	37.9 <sup>±</sup> 5.1	39.4 <sup>±</sup> 3.3	38.2 ±3.2	37.5 <sup>±</sup> 1.6

TABLE 5c

BLOOD VOLUME ML./KG.

No. No.	G			Weeks		
An. No.	Group	0	4	10	18	28
5	Control	48.8	48.0	47.1	51.9	50.1
6	п	52.6	55.7	53.7	52.2	49.5
3		45.1	45.8	47.5	49.7	-
9	"	46.4	46.6	48.2	48.2	-
Mean		48.2 <sup>+</sup> 3.3	49.0 -4.5	49.1 <sup>+</sup> 3.1	51.2 -3.0	49.8 -0.6
7	Infected	52.4	56.5	51.9	49.3	53.2
8		48.8	50.2	49.7	52.8	50.4
8в		47.7	47.1	51.5	47.5	-
10	"	50.4	47.5	45.5	49.1	
Mean		49.8 <sup>+</sup> 2.0	50.3 +4.3	49.7 <sup>+</sup> 2.9	49.7 +2.2	51.8 <sup>+</sup> 2.8

TABLE 7c

RED CELL VOLUME ML./KG.

No. Ma	Chaus			Weeks		
An. No.	Group	0	4	10	18	28
5	Control	17.6	14.4	16.0	18.2	16.5
6	u	21.0	16.7	18.7	19.9	17.3
3	"	16.5	15.8	15.0	18.1	-
9	"	18.6	17.9	15.7	18.3	-
Mean		18.4 <sup>±</sup> 1.9	16.2 <sup>±</sup> 1.5	16.3 ±1.6	18.6 <sup>±</sup> 0.9	16.9 ±0.8
7	Infected	19.4	11.9	11.4	11.8	14.9
8	"	20.5	11.8	10.4	10.0	13.7
88	"	21.2	11.1	8.7	10.5	-
10	"	17.1	15.0	10.5	13.7	-
Mean		19.5 -1.8	12.4 ±1.7	10.3 -1.1	11.5 -1.7	14.3 ±1.2

TABLE 8c

IRON UTILIZATION IN T. CONGOLENSE INFECTED CATTLE (\* 2S.E.)

Group	Serum Iron (µg/loòml.)	Total Iron binding Cap. (µg/looml.)	Plasma Iron transport Mg./Day/looml.
	153.0-20.4	334.2 <sup>+</sup> 39.6	0.86±.22
	241.4 <sup>+</sup> 25.0	412.4 <sup>+</sup> 26.6	2.50-80
	85.00-25.0	203.5 <sup>+</sup> 29.6	0.95+.24
	26.70-4.7	150.0-45.7	0.2704
(treated 20 wks.)	101.00-26.0	167.0-10.0	0.8814
	141.7 <sup>±</sup> 54.5	384.3 <sup>+</sup> 53.7	1.27±.34
61 wks. (treated 20 wks.)	136.0-32.0	377.0-22.0	1.06-10

### SECTION D

### IMMUNITY IN CATTLE TO TRYPANOSOMA CONGOLENSE

A total of 42 Hereford cattle of various ages were infected with the trans mara strain of T. congolense and observed for evidence of an age resistance. Results showed that eight of nine cattle infected at one year of age or less survived the infection without treatment. Two animals of eight in the age range of one to two years also survived the infection. All 25 animals whose ages ranged from two to five years either succumbed to the infection or had to be treated because of the severity of the disease. When the young animals, which needed no treatment to survive, were rechallenged at periods out to one year after the last observation of patent parasitemia, they appeared to be completely refractive to infection. The older animals which required Berenil treatment to survive were also rechallenged at intervals after therapy. Three animals infected for 49 to 75 days before treatment were rechallenged 198 to 296 days later. Extensions in prepatent periods ranged from five to 13 days when compared to controls and the resulting infections were of a relapsing nature followed by self cure. Effects of this disease on clinical parameters were minimal. One animal infected for 196 days and rechallenged 501 days later had a prepatent period of 14 days as compared to five days for controls. This animal developed a brief relapsing infection followed by self cure. Animals which were infected for periods of 41 to 77 days, received treatment, and were then rechallenged from 600 to 900 days later, showed some resistance to infection. Prepatent periods were extended from one to three days over those of control animals and although the resulting disease was severe, one of four animals self cured without treatment. When animals which had self cured secondary challenges were rechallenged at periods out to two years later, they were completely refractory to homologous challenge from mice. These animals developed brief infections when challenged with the relapse variants collected from a bovine chronically infected with the homologous strain. Prepatent periods were extended however, and the infections were rapidly eliminated without severe clinical disease. Since the bovine developed strong resistance to a challenge of syringe passed trypanosomes we have recently initiated a study to determine whether or not they are also immune when the homologous strain of parasite is transmitted by fly bite. Tsetse flies (Glossina morsitans) were infected by daily exposure to infected guinea pigs or bovines for a period of two weeks. Two weeks after the last exposure, the flies were fed on immune and non-immune cattle. All control animals (nine) have developed high levels of persistant parasitemia. Six immunized cattle have developed low level relapsing infections while the remaining nine immunized animals have not developed detectable parasitemias as yet. Significant anemia, leucopenia and thrombocytopenia have developed in all controls; however, immunized animals have not shown any severe clinical disease.

### SECTION E

# IMPROVEMENTS TO THE LABORATORY FACILITY AND PREPARATION OF NEW REAGENTS

While a strong immunity to blood forms was apparent in calves who had self cured T. congolense infections and in adults who were repeatedly infected and cured, the immunity needed to be tested against metacyclic challenge from tsetse flies. We were given extra facilities at the Veterinary Research Laboratories (1,000 sq. ft.) which we reconditioned into an insectory, laboratory, small animal room and exposure room for large animals. The facility has been double screened and equipped with insectocutors. The large animal exposure room contains a stanchion whereby cattle can be exposed to fly bites indoors. Arrangements were made through Dr. Anthony Jordan at Bristol University, England, to receive 200 G. morsitans pupae per month on a regular basis. At the present time, although we are still receiving the pupae from England we are collecting approximately 1,000 pupae a month from our own rearing colony. The Trans-Mara I strain of T. congolense which had been used throughout our immunization studies was readily transmitted by the  $\underline{G}$ . morsitans flies and we were able to initiate the metacyclic challenge experiments which are now in progress.

During the past year, bovine serum has been fractionated and the immunoglobulins purified. These reagents have been used to immunize goats who will provide antisera which will be conjugated with fluorescin isothiocyanate and used in immunopathological investigations.

PROJECT 3M161102BS01 RESEARCH ON MILITARY DISEASES

Work Unit 138 Vaccine Development in Trypanosomiasis

### Literature Cited

### References:

- 1. Calwell, H.G., 1937. The pathology of the brain in Rhodesian trypanosomiasis. Trans. Roy. Soc. Trop. Med. Hyg. 30:611
- 2. Gray, A.R., 1970. A study of the antigenic relationships of isolates of <u>Trypanosoma brucei</u> collected from a herd of cattle kept in one locality for five years. J. Gen. Microbiol. <u>62</u>:301
- 3. Gray, A.R., 1972. Variable agglutinogenic antigens of <u>Trypanosoma gambiense</u> and their distribution among isolates of the trypanosome collected in different places in Nigeria. Trans. Roy. Soc. Trop. Med, and Hyg. 66:263
- 4. Gray, A.R., 1975. A pattern in the development of agglutinogenic antigens of cyclically transmitted isolates of <u>Trypanosoma</u> gambiense. Trnas. Roy. Soc. Trop. Med. Hyg. 69:131
- 5. Manuelidis, E.C., Robertson, D.H., and Amberson, J.M., 1965.  $\underline{\text{Trypanosoma rhodesiense}}$  encephalitis: Clinicopathologic study of five casts of encephalitis and of MELB hemorrhagic encephalopathy. Acta Neuropathol. (Berlin)  $\underline{5}$ :1965
- 6. Ormerod, W.E., 1970. The choroid plexus in African sleeping sickness. Lancet 10:777
- 7. Soltys, M.A., 1957. Immunity in trypanosomiasis 1. Neutralization reaction. Parasitology <u>47</u>:375

	AND TECHNOLOGY	Y WORK UNIT S	UMMARY	DA C	A 6436	1	77 10 0	1		CONTROL SYMBOL R&E(AR)636
76 10 01	D. Change	S. SUMMARY SCTY	4. WORK SECURITY	NA NA	DING	NL	P'N INSTA'N	OL SPECIFIC CONTRACTOR	ACCESS	A WORK UNIT
10. NO./CODES:*	PROGRAM ELEMENT	PROJECT	NUMBER	TASK AREA NUMBER WORK UNIT NUMBER						
& PRIMARY	61102A	3M16110	2BS01	0	00 139					
b. CONTRIBUTING						8				
CX KGCXBCBCXDCX	CARDS 114F									
the state of the s	ial Genetics		my							
010100 Mic										
13. START DATE		14. ESTIMATED COMP	PLETION DATE	IS FUND	NIG AGENCY			16. PERFORM		
63 08		CONT		DA				C. In-	House	2
17. CONTRACT/GRANT				10. RESC	OURCES ESTIM	ATE	& PROFESSI	ONAL MAN YRS	h Fue	DS (In thousands)
& DATES/EFFECTIVE:	NA	EXPIRATION:			PRECEDING					
& NUMBER:				FISCAL 77			3		2	271
C TYPE:		& AMOUNT:		YEAR	CURRENT					
& KIND OF AWARD:		f. CUM. AMT.			78		4		34	1
19. RESPONSIBLE DOD O	PREAMIZATION				ORMING ORGA					
MAME: Walter	Reed Army Ins	stitute of	Research	NAME:	Walter	Re	ed Army	Institu	ite o	Research
					Div of	CD	&I			
ADDRESS: Washin	gton, DC 200	012		ADDRESS	· Washi	ng	ton, DC	20012		
								I U.S. Academic	[ne!/hutlan	
RESPONSIBLE INDIVIDU	AL			NAME:			, L.S.			
	d, COL Garris	son		TELEP	HONE: 202	-5	76-2230			
TELEPHONE: 202	-576-3551			_	SECURITY AC		- Street			
21. GENERAL USE				ASSOCIAT	TE INVESTIGAT	TORS	Wohlh	ieter,	.A.	
Foreign in	telligence no	t consider	ed	NAME:						DA
W + 5 × ma = 0 + (n 4				NAME:						
	EACH with Socuelty Classific	(0)	Vaccine; (	U) En	teric B	act	teria;	(U) Anti	gens	
	nce; (U) Salm									
23. TECHNICAL OBJECT	IVE. 24 APPROACH, 25.	PROGRESS (Fumial I	idividual paragraphe ide	entitled by	number. Preced	. test	of sech with S	curity Classific	tion Code	)

- 23. (U) Definition in genetic and molecular terms of the properties of gene transfer antigenicity, and virulence of pathogenic enteric bacteria which because of the  ${\rm i} {\rm r}$
- disease producing capabilities, are of importance to military medicine concerned with the prevention and treatment of such infections in Army personnel. We anticipate that it will be possible to genetically modify enteric bacteria to any desired antigenic structure and pathogenicity to serve as vaccine strains or as tools to study the infectious process.
- 24. (U) Use of genetic recombination between strains of enteric bacteria. Where possible, the genetic results are extended to include study of the informational macromolecules involved.
- 25. (U) 76 10-77 09 Live Salmonella typhi administered intraperitoneally, acetonekilled S. typhi administered intraperitoneally, and live S. typhi given orally, with their effectiveness decreasing in that order, protected Swiss white mice against death from challenge with a virulent S. typhimurium hybrid expressing S. typhi antigens. The structural genetic determinants of the Vi antigen of S. paratyphi C were shown to be allelic with those of the S. typhi Vi antigen, and their expression was found to be affected by the viaA gene in the same manner as expression of the S. typhi determinants is affected by this gene. An Escherichia coli K-12 mutant that limits the growth of coliphage lambda was isolated and characterized. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 76 - 30 Sep 77.

Project 3M161102BS01 BASIC RESEARCH ON MILITARY DISEASES

Work Unit 139 Microbial Genetics and Taxonomy

Investigators.

Principal: Louis S. Baron, Ph.D

Associate: J. A. Wohlhieter, Ph.D.; E. M. Johnson, Ph.D.; C. A. Life; N. J. Snellings, M. S.; SP4 J.L.

Vickroy, A.B.; SP4 A.B. Corpuz, B.S.; SP4 T.

Casey, A.B.; CPT D. J. Kopecko, Ph.D

# Description.

- 1. Live <u>Salmonella typhi</u> administered intraperitoneally, acetone-killed <u>S. typhi</u> administered intraperitoneally, and live <u>S. typhi</u> given orally, with their effectiveness decreasing in that order, protected <u>Swiss white mice against death from challenge with a virulent <u>S. typhimurium hybrid expressing <u>S. typhi</u> antigens.</u></u>
- 2. The structural genetic determinants of the Vi antigen of Salmonella paratyphi  $\underline{C}$  were shown to be allelic with those of the  $\underline{S}$ . typhi Vi antigen at the locus viaB, and their expression was found to be affected by the viaA gene in the same manner as expression of the  $\underline{S}$ . typhi determinants is affected by this gene.
- 3. An Escherichia coli K-12 mutant that limits the growth of coliphage lambda was isolated and characterized. This mutation is not found in S. typhi, which inhibits growth of lambda through a similar mechanism, controlled by a different, unlinked gene locus previously characterized.

### Progress.

- 1. Effectiveness of parenteral and oral typhoid vaccination in mice challenged with a Salmonella typhi-S. typhimurium hybrid.
- a. In previous reports (Annual Report, WRAIR, 1973, 1974, 1976)we have described the development and employment of an assay system for differentiating the protective activities of various typhoid vaccines. It uses Swiss Webster white mice as the test animals and mouse-virulent Salmonella typhimurium hybrids that express S. typhi antigens as the challenge strains. In our earlier studies, we showed that this system can demonstrate differences among various kinds of typhoid vaccines with respect to their ability to confer protection against death of the animals, and we provided evidence that the Samonella somatic antigens are important in conferring this protection, whereas the Vi antigen appears to play no significant role. The vaccines examined in those studies were nonliving and, in all cases, the immunizing doses were administered intraperitoneally (i.p.). In the present study, we have used our assay system to investigate the protective capabilities of living as well as acetone-treated (AK) S. typhi vaccines administered both orally and i.p. We also have examined a purified S. typhi Vi antigen preparation administered by both routes.

- b. The AK vaccine was prepared from <u>S</u>. <u>typhi</u> as described previously (Annual Report WRAIR 1973). The purified Vi antigen, prepared from <u>S</u>. <u>typhi</u> strain 59, was supplied by S. Marcus of the University of Utah, Utah Medical Center, Salt Lake City. Swiss Webster white mice (50 per group), HPB strain, random bred, 16 to 18 g, were inoculated i.p. with 0.5 ml of the AK vaccine (equivalent to 5 x  $10^8$  organisms) or with 0.5 ml of a live vaccine ( $10^7$  organisms) prepared from a 16-h culture of S. typhi TY2. Vi antigen vaccine was administered in two doses of 0.5 ml ( $500~\mu g/dose$ ), with a 1-week interval between the first and second injection. The same doses of vaccines AK, live, and Vi were administered orally to other groups of mice. Animals vaccinated with AK and Vi vaccines either i.p. or orally were challenged i.p. 2 weeks after the last vaccination with 2,500 organisms (0.5 ml) of <u>S</u>. typhimurium hybrid H42. This hybrid expresses the <u>S</u>. typhi antigens 9, 12, Vi, and d and has a mean lethal dose of less than 50 organisms. Animals injected with live vaccines either i.p. or orally were challenged after 5 weeks in the same manner.
- c. From the results of these experiments, as presented in Table 1, it is apparent that the best protection against death from the hybrid challenge organism was afforded by the live and AK vaccines when administered i.p. The live vaccine was slightly better than the AK vaccine in this comparison (P<0.05). Also, oral administration of the live cells afforded significant protection against death whereas oral administration of the AK vaccine did not. It might be worth noting, however, that the degree of protection afforded by the live cells administered orally did not, in this sytem, match that afforded by the AK vaccine administered i.p. Whether or not a similar situation would be observed in immunizing against human typhoid fever with living, attenuated, orally administered vaccines, as opposed to killed vaccines parenterally administered, remains to be determined.
- - 2. Genetic basis of Vi antigen expression in Salmonella paratyphi C.
- a. The antigen termed Vi is expressed by several organisms of the family Enterobacteriaceae, but only in Salmonella typhi has the genetic basis of this expression been examined. There, we have shown previously (2,3) that determinants of the antigen occupy two widely separated chromosomal gene loci, designated viaA and viaB. The S. typhi viaB locus, situated near the determinant of adenine biosynthesis, purA, is occupied by the genes which, when introduced into S. typhimurium or Escherichia coli, result in the expression of the Vi antigen in those organisms. It therefore appears to be the site at which the primary structural

determinants of the antigen are located. The <u>viaA</u> locus is situated near the gene determining histidine biosynthesis, <u>his</u>. Mutation of a gene at the <u>viaA</u> locus of <u>S</u>. <u>typhi</u> results in loss of Vi antigen expression even though the genes at the <u>viaB</u> locus remain functional. The specific function of the <u>viaA</u> gene is not known, but this determinant is present also in other enteric bacteria, such as <u>S</u>. <u>typhimurium</u> and <u>E</u>. <u>coli</u> which do not normally express the Vi antigen. Thus, introduction of the native, functional <u>viaA</u> locus of <u>S</u>. <u>typhimurium</u> or <u>E</u>. <u>coli</u> into a <u>viaA</u> mutant of <u>S</u>. <u>typhi</u> restores that organisms's ability to express the antigen.

- b. In addition to its occurrence in S. typhi, the Vi antigen is found also in strains of S. dublin, in certain serotypes of Citrobacter, and in S. paratyphi C (S. hershfeldii). Although serologically identical, the antigens expressed by these organisms appear to differ among each other in varying degrees, both chemically and immunologically. Thus, Edwards and Ewing (1) have pointed out, from the results of immunoelectrophoretic analysis, that of the six identified, cathodically migrating fractions of the S. typhi Vi antigen, four are shared by the Vi antigen of the Citrobacter serotypes, whereas the S. paratyphi C Vi antigen consists of just two fractions that are related to two of those shared by the S. typhi and Citrobacter antigens. Nevertheless, it seemed to us likely that their genetic determinants should be related to each other in the same way as are the genetic determinants of the diverse somatic and flageller antigens expressed among the Enterobacteriaceae, i.e. as members of a series of allelic genes occupying the same chromosomal locus in each organism. Having previously adapted a strain of S. paratyphi C to serve as an Hfr donor (E.M. Johnson, unpublished data), we were afforded the opportunity of examining this proposition, at least with respect to the Vi antigen determinants of S. paratyphi C and S. typhi.
- c. We previously established the location of the S. typhi viaB locus between the metA and purA loci, somewhat more closely linked to purA than to metA. Thus, it occupies a position very close to the determinants of melibiose utilization (mel). On the assumption that the structural genetic determinants of the S. paratyphi C Vi antigen would occupy the same chromosomal locus in that organism as in S. typhi, we mated the S. paratyphi C Hfr, WR4060 with the S. typhimurium recipient WR5001, selecting for hybrids receiving the mel marker of the donor. Not surprisingly, Vi antigen expression occurred in 72 of 100 Me1+ selected hybrids. The neighboring metA donor marker was inherited by only 18% of them. When the metA gene was used as the selected marker in this cross, 20% of the hybrids inherited the Vi antigen determinant and 27% inherited the donor mel marker. Of the 28 Met selected hybrids that expressed either the mel or via determinants, 19 expressed both of those markers. These results, as well as the nature and number of the hybrid classes formed in these matings, are shown in Table 2 and are indicative of a very close linkage of the mel and via genes. The appearance

of 8  $\underline{\text{metA}}$ ,  $\underline{\text{mel}}$  hybrids, as opposed to only one  $\underline{\text{metA}}$ ,  $\underline{\text{via}}$  hybrid among those selected for receipt of the donor  $\underline{\text{metA}}$  gene suggests that the genes order is  $\underline{\text{metA}}$ ,  $\underline{\text{mel}}$ ,  $\underline{\text{via}}$ .

- d. Having established that the newly mapped via determinant of S. paratyphi C occupies a chromosomal location apparently similar to that previously determined for the S. typhi viaB genes, we then set up the following experiment to demonstrate the allelic nature of these genes. We employed the lone Met<sup>+</sup>, Vi antigen expressing, Mel<sup>-</sup> S. typhimurium WR5001 hybrid fathered by the S. paratyphi C Hfr WR4060 as the recipient in a mating with S. typhi Hfr WR4000. With each parent in this cross expressing a Vi antigen of different genetic origin, we reasoned that if their via determinants did not occupy a common Jocus, loss of Vi antigen expression should be observed occasionally among the Mel<sup>+</sup> selected hybrids. In other words, some hybrids would be expected to lose their S. paratyphi C via genes by recombination while also failing to inherit the viaB determinants of the S. typhi donor. However, in examining over 500 Mel+ selected hybrids from this cross, we did not observe any in which loss of Vi antigen expression occurred. The indication, therefore, is that the structural determinants of the Vi antigen of S. paratyphi C occupy the same chromosomal locus in that organism as the one at which, in S. typhi, the viaB genes reside.
- e. We were able to demonstrate a role for the  $\underline{viaA}$  determinant in expression of the  $\underline{S}$ .  $\underline{paratyphi}$   $\underline{C}$  Vi antigen by using a non-Vi expressing  $\underline{S}$ .  $\underline{typhi}$  Hfr, WR4013, with functional  $\underline{viaB}$  genes but a mutant  $\underline{viaA}$  determinant, to transfer  $\underline{his}$  and the mutant  $\underline{viaA}$  gene, to an  $\underline{S}$ .  $\underline{typhimurium}$  WR5001 Me1 hybrid expressing the  $\underline{S}$ .  $\underline{paratyphi}$   $\underline{C}$   $\underline{viaB}$  genes. In this cross, 25% of the His selected hybrids lost their ability to express the Vi antigen as the result of inheriting the mutant  $\underline{viaA}$  gene of the  $\underline{S}$ .  $\underline{typhi}$  donor. A similar result (23% loss of Vi antigen expression) was observed when a Me1  $\underline{S}$ .  $\underline{typhimurium}$  WR5001 hybrid expressing the  $\underline{S}$ .  $\underline{typhi}$   $\underline{viaB}$  genes served as the recipient with this Hfr.
- 3. Cooperative effects of bacterial mutations affecting lambda N gene expression.
- a. In coliphage lambda, the N gene specifies a protein which regulates the expression of most lambda functions. This regulation takes place during genetic transcription, and a host factor synthesized by the bacterium which interacts with the N protein is required. Various  $\underline{E}$ .  $\underline{\operatorname{coli}}$  mutants limit lambda phage growth by inhibiting the action of the N gene protein. We have previously shown that  $\underline{S}$ .  $\underline{\operatorname{typhi}}$  limits the growth of lambda as well as other coliphages. This result was obtained with  $\underline{E}$ .  $\underline{\operatorname{coli}}-\underline{S}$ .  $\underline{\operatorname{typhi}}$  hybrid WR4255 which adsorbs coliphage lambda having acquired the  $\underline{E}$ .  $\underline{\operatorname{coli}}$  genes necessary for synthesis of the cell surface receptor for phage attachment. The  $\underline{S}$ .  $\underline{\operatorname{typhi}}$  hybrid limits growth of lambda by interfering with the action of the N protein in a manner similar to that exhibited by an  $\underline{E}$ .  $\underline{\operatorname{coli}}$  mutant also characterized. Both  $\underline{S}$ .  $\underline{\operatorname{typhi}}$  and the  $\underline{E}$ .  $\underline{\operatorname{coli}}$  mutant were shown to be defective in a bacterial locus involved

in N expression, the  $\underline{\text{nusA}}$  locus, which maps at minute 68 of the  $\underline{\text{E}}$ .  $\underline{\text{coli}}$  K-12 chromosome.

- b. A number of additional E. coli mutants have recently been isolated which also act to limit the growth of phage lambda by inhibiting the expression of the N gene regulatory function. Most of these mutants, although phenotypically similar to the nusA mutant, did not map at the nusA locus. One of these mutants with the Nus phenotype was chosen for intensive study. This mutant, referred to as nus B-5 was used as the recipient in crosses with different E. coli Kl2 Hfr donors and hybrids were selected using markers distributed around the K12 chromosomal map. The results established that the nusB locus was closely linked to the locus for lactose (<u>lac</u><sup>+</sup>) utilization. Thus, the <u>nusB</u><sup>+</sup> marker replaced the <u>nusB</u><sup>-</sup> allele of the recipient in 75-95% of the <u>Lac</u><sup>+</sup> recombinants when HfrH or HfrC, both transferring lac+ as an early marker but from different directions, were used as donors. When P4X-6, which transfers lac as a terminal marker, was used as the donor, replacement of the nusB allele by the nusB<sup>+</sup> allele of this donor dropped to less than 5%. This result established the location of nusB between the origin of HfrC and Hfr P4X-6, a six minute segment of the K12 chromosome.
- c. More precise mapping of the <u>nusB</u> marker was accomplished by transduction experiments with phage Pl <u>vir</u>. Lysates grown on the <u>nusB</u> mutant were used to infect a Nus<sup>+</sup> recipient, WR2099 (lac <u>proC purE</u>), with subsequent selection for <u>lac</u><sup>+</sup>, <u>proC</u><sup>+</sup>, and <u>purE</u><sup>+</sup> single marker transductants. The results show that the <u>nusB</u> marker was not contransduced with either <u>lac</u><sup>+</sup> or <u>purE</u><sup>+</sup>, but was contransduced with <u>proC</u><sup>+</sup> at a frequency of approximately 2%. An experiment was also performed, using a Pl lysate grown on a mucoid (lon) strain, to select <u>proC</u><sup>+</sup> transductants to determine the order of the <u>nusB</u> locus with respect to <u>proC</u> and <u>lon</u>. The results indicated the order of these loci to be <u>proC</u> lon <u>nusB</u>, with <u>nusB</u> mapping near minute 11, since cotransduction of <u>proC</u><sup>+</sup> and <u>lon</u> was 2%, while the cotransduction of <u>proC</u><sup>+</sup> and <u>nusB</u> was 2%.
- d. Even though the  $\underline{\text{nusA}}$  and  $\underline{\text{nusB}}$  loci map at distant sites on the  $\underline{\text{E.}}$   $\underline{\text{coli}}$  chromosome, both mutant types exhibit strikingly similar effects on  $\underline{\text{lambda}}$  phage growth. In order to determine the effect of the combination of the  $\underline{\text{nusA}}$  and  $\underline{\text{nusB}}$  mutations on lambda growth, we constructed a double  $\underline{\text{nus}}$  mutant ( $\underline{\text{nusA}}$  and  $\underline{\text{nusB}}$ ) and found this double  $\underline{\text{nus}}$  mutant to be far more restrictive on lambda growth than either of the single  $\underline{\text{nus}}$  mutants.
- e. We have previously demonstrated that  $\underline{S}$ .  $\underline{typhi}$  does not carry the  $\underline{nusA}$  allele, as is also the case with  $\underline{S}$ .  $\underline{typhimurium}$  strains. To determine if  $\underline{Salmonella}$  carry the  $\underline{nusB}^+$  allele, we constructed intergeneric hybrids by crossing  $\underline{E}$ .  $\underline{coli}$  Hfr  $\underline{P4X-6}$  with  $\underline{S}$ .  $\underline{typhi}$  recipient WR4204 and selecting for  $\underline{ara}^+$  recombinants. A stable  $\underline{E}$ .  $\underline{coli-S}$ .  $\underline{typhi}$  hybrid was examined which acquired the  $\underline{nusA}^+$  as well as the  $\underline{malB}^+$  and  $\underline{malA}^+$  loci required for lambda adsorption, but retained the  $\underline{\underline{Salmonella}}$  region encompassing the  $\underline{nusB}$  locus. Since this  $\underline{S}$ .  $\underline{typhi}$  hybrid permits lambda

growth, we conclude that  $\underline{S}$ .  $\underline{typhi}$  carries the  $\underline{nusB}^+$  allele. We were also able to transfer the  $\underline{nusB}^-$  allele from  $\underline{E}$ .  $\underline{coli}$  to  $\underline{S}$ .  $\underline{typhi}$  by selecting for  $\underline{lac}^+$  hybrids. These hybrids were either unstable  $\underline{lac}^+/\underline{lac}^-$  diploids expressing the  $\underline{nusB}^+$  trait or stable  $\underline{lac}^+$ hybrids which had integrated the  $\underline{lac}^+$  K12 chromosomal region. Such stable  $\underline{lac}^+$   $\underline{S}$ .  $\underline{typhi}$  hybrids were found to express the  $\underline{Nus}^-$  phenotype, indicating that they had integrated the  $\underline{nusB}^-$  allele transferred from the K12 donor.

### Summary

- 1. A mouse virulent <u>Salmonella</u> <u>typhimurium</u> hybrid expressing the <u>S. typhi</u> antigens 9, 12, Vi and d was used as a challenge organism in <u>Swiss white mice to test the ability of acetone-treated S. typhi</u>, live <u>S. typhi</u>, and purified Vi antigen, each administered orally and intraperitoneally, to protect the animals against death. Live <u>S. typhi</u> administered intraperitoneally, acetone-killed <u>S. typhi</u> administered intraperitoneally, and live <u>S. typhi</u> given orally, with their effectiveness decreasing in that order, <u>conferred</u> protection in this system.
- 2. Analysis of hybrids formed in the cross between an S. paratyphi Hfr and an S. typhimurium recipient indicated that the structural genetic determinants of the S. paratyphi C Vi antigen are located closely adjacent to the mel determinant, between this marker and purA. A similar location was indicated previously for the structural genetic determinants of the S. typhi Vi antigen (the viaB locus). Mating experiments with a Vi antigen expressing S. typhi Hfr and an S. typhimurium hybrid recipient expressing the Vi antigen of S. paratyphi C yielded no recombinants in which loss of Vi antigen expression occurred, indicating that the chromosomal locus occupied by the genetic determinants of the S. paratyphi C Vi antigen is the same one at which, in S. typhi, the viaB genes reside. Introduction of a mutant S. typhi viaA gene into an S. typhimurium hybrid expressing the Vi antigen, as the consequence of prior receipt of the S. paratyphi C viaB determinants, resulted in that hybrid's loss of Vi antigen expression, demonstrating that the viaA determinant plays a role in Vi antigen expression in S. paratyphi C, as well as in S. typhi.
- 3. An  $\underline{E}$ .  $\underline{\operatorname{coli}}$  mutant which limits the growth of phage lambda by inhibiting the expression of the N gene regulatory function was isolated and characterized. The mutation involved maps near minute 11 of the  $\underline{E}$ .  $\underline{\operatorname{coli}}$  chromosome and dominance tests show that the mutant allele is recessive. We conclude therefore that the locus involved normally codes for a function necessary for N gene expression. Another mutant with a similar phenotype has been mapped at minute 68; this mutant is called Nus (N utilization substance). We have named the locus at minute 68  $\underline{\operatorname{nusA}}$ , and the locus at minute 11  $\underline{\operatorname{nusB}}$ . Although the  $\underline{\operatorname{nusA}}$  allele is not found in  $\underline{S}$ .  $\underline{\operatorname{typhi}}$  ( $\underline{\operatorname{nusA}}$ ), we have found the  $\underline{\operatorname{nusB}}$  allele to be present in  $\underline{S}$ .  $\underline{\operatorname{typhi}}$  ( $\underline{\operatorname{nusB}}$ ).

Table 1. Effectiveness of AK, live, and Vi antigen vaccines in mice challenged with  $\underline{S}$ .  $\underline{typhimurium}$  hybrid H42

Vaccinated with	Vaccinating dose	Route	Interval between last vac- cination and chal- lenge (weeks)	
AK	5 x 10 <sup>8</sup> cells	i.p.	2	30/50 <sup>a</sup>
AK	$5 \times 10^8$ cells	Oral	2	7/50
TY2 live	$10^{7}$ cells	i.p.	5	40/50 <sup>a</sup>
TY2 live	10 <sup>7</sup> cells	Oral	5	21/50 <sup>b</sup>
Vi	2 x 500 μg	i.p	2	9/50
Vi	2 x 500 μg	Oral	2	3/50
Control				8/50

<sup>&</sup>lt;sup>a</sup>Significantly better (P < 0.005) than the controls <sup>b</sup>Significantly better (P < 0.01) than the controls.

Table 2. Unselected marker inheritance of hybrids formed in crosses between S. paratyphi C Hfr WR4060 and S. typhimurium recipient WR5001a  $\underline{\mbox{C}}$ 

Donor Marker Selected	Hybrid Classes	No.	Summation of Unselected Marker Inheritance
	mel, via	56	<u>via</u> 72%
me1	me1	26	
	mel, metA, via	16	<u>metA</u> , 18%
	mel, metA	2	
	metA	72	<u>mel</u> , 27%
	metA, mel, via	19	
metA	metA, mel	8	<u>via</u> , 20%
	metA, via	1	

<sup>&</sup>lt;sup>a</sup>Markers listed are those inherited from the Hfr.

Project 3M161102BS01 BASIC RESEARCH ON MILITARY DISEASES

Work Unit 139 Microbial Genetics and Taxonomy

### Literature Cited.

### References:

- 1. Edwards, P.R. and Ewing, W.H.: Identification of Enterobacteriacae. Burgess Publishing Co., Minneapolis. 1972.
- 2. Johnson, E.M., Krauskopf, B., and Baron, L.S.: Genetic mapping of Vi and somatic antigenic determinants in <u>Salmonella</u>. J. Bacteriol. 90:302-308, 1965.
- 3. Johnson, E.M., Krauskopf, B., and Baron, L.S.: Genetic analysis of the Via-his chromosomal region in <u>Salmonella</u>. J. Bacteriol. 92:1457-1463, 1966.

### Publications:

- 1. Baron, E.S., Saz, A.K., Kopecko, D.J. and Wohlhieter, J.A.: Transmissible B-lactamase plasmid in Neisseria gonorrhoeae. Abst. Ann. Meeting, A.S., p.75, 1977.
- 2. Baron, E.S., Saz, A.K., Kopecko, D.J. and Wohlhieter, J.A.: Transfer of plasmid-borne beta-lactamase in Neisseria gonorrhoeae. Antimicrob. Agents Chemother. 12:270-280, 1977.
- 3. Baron, L.S, Life, C.A., Gemski, P., and Yamamoto, N.: Expression of <u>Salmonella</u> somatic antigen 0-1 by <u>Escherichia</u> <u>coli</u> K-12. Abst. Ann. Meeting, A.S.M., p. 76, 1977.
- 4. Cohen, S.N. and Kopecko, D.J.: Structural evolution of bacterial plasmids: role of translocating genetic elements and DNA sequence insertions. Fed. Proc. 35:2031-2036, 1976.
- 5. Diena, B.B., Ryan, A., Wallace, R., Johnson, E.M., Baron, L.S. Ashton, F.E.: Effectiveness of parenteral and oral typhoid vaccination in mice challenged with a <u>Samonella</u> <u>typhi-Samonella</u> <u>typhimurium</u> hybrid. Infect. Immun. 15:997-998, 1977.
- 6. Friedman, D.I., Baumann, M. and Baron, L.S.: Cooperative effects of bacterial mutations affecting lambda N gene expression. I. Isolation and characterization of a <u>nusB</u> mutant. Virology <u>73</u>:119-127, 1976.
- 7. Kopecko, D.J., Brevet, J. and Cohen, S.N.: Involvement of multiple translocating DNA segments and recombinational hotspots in the structural evolution of bacterial plasmids. J. Mol. Biol. 108:333-360, 1976.

- 8. Macrina, F.L., Reider, J.L., Virgili, S.S. and Kopecko, D.J.: Survey of the extrachromosomal gene pool of <u>Streptococcus</u> <u>mutans</u>. Infect. Immun. 17:215-226, 1977.
- 9. Moseley, S.L., Kopecko, D.J., and Gemski, P.: Genetic and physical characterization of the Vir plasmid of Escherichia coli. Abst. Ann. Meeting, A.S.M., p. 366, 1977.
- 10. Snellings, N.J., Johnson, E.M., and Baron, L.S.: Genetics of Vi antigen expression in <u>Salmonella paratyphi</u> C. Abst. Ann. Meeting, A.S.M., p. 76, 1977.
- 11. Snellings, N.J., Johnson, E.M., and Baron, L.S.: Genetic basis of Vi antigen expression in <u>Salmonella paratyphi</u> C. J. Bacteriol. <u>131</u>:57-62, 1977.
- 12. Wohlhieter, J.A., Johnson, E.M., and Baron, L.S.: Isolation and characterization of plasmids from <u>Salmonella</u> strains fermenting lactose and sucrose. Abst. NATO Adv. Study Inst., Athens, Greece, App. W, 1976.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY					DA OA 6450		77 10 01		DD-DR&E(AR)636		
1. DATE PREV SUMPRY	4. KIND OF SUNMARY		. WORK SECURITY	7. REGRA	DINGS BA D	SO'N INSTA'N	OB SPECIFIC	C DATA-	S. LEVEL OF SUM		
76 10 01	D. Change	PROJECT	U	N/	REA NUMBER	NL Stres -		□ mo	A WORK UNIT		
PRIMARY	1 61102A	3M161102B		O(			WORK UNIT NUMBER				
b. CONTRIBUTING	+ 011021	3F1101102B	301	1-00		140					
c. CONTRIBUTING	CARDS 114F										
	Security Classification Code	y•									
	ry Hematology										
008800 Lif		002600 Biol			Clinica	1 Medic	ine				
13. START DATE	START DATE 14. ESTIMATED COMPLETION DATE				NG AGENCY			MANCE MET			
58 05		CON	1	DA		4		n-House			
				10. RESO	PRECEDING	E A PROFE	A PROFESSIONAL MAN YRS L		NDS (In thousands)		
& DATES/EFFECTIVE	NA NA	EXPIRATION:	EXPIRATION:		FISCAL 77		10		404		
C TYPE:		d AMOUNT:		YEAR CURRENT		+		+	<del>                                     </del>		
e. KIND OF AWARD:		f. CUM. AMT.		1 1	78		10		397		
19 RESPONSIBLE DOD	ORGANIZATION			20. PERF	PHING ORGANI	MOITAS					
NAME:*				NAME:	dalter R	eed Arm	v Instit	tute o	f Research		
	d Army Institu		arch	Manual Walter Reed Army Institute of Research Division of Medicine							
ADDRESS:* Wa	shington, DC	20012		ADDRESS:* Washington, DC 20012							
				1							
RESPONSIBLE INDIVID	NIAI			PRINCIPAL INVESTIGATOR (Furnish SEAN IL U.S. Academic Institution)  NAME: HAUT. LTC M.J.							
	ind, COL G.			TELEPHONE: 202-576-3358							
TELEPHONE: 202-576-3551					SOCIAL SECURITY ACCOUNT NUMBER:						
21. GENERAL USE					ASSOCIATE INVESTIGATORS						
Foreign intelligence not considered					HAME: Dimond, LTC R.C. DA						
22. KEYWORDS /Freced	BACH with Socuelty Classifi	(cetter Code)		NAME:							
(II) Coagul	lation: (U) M	alaria: (U	) Blood;	(U) A	nemia						
23. TECHNICAL OBJEC	TIVE. 24 APPROACH, 25	PROGRESS (Furnish in	dividual paragraphs is	dentitled by	number. Precede i						
	define the he										
importance, and of trauma, burns and shock, and to identify modalities to restore											
hemostasis.											
24. (U) Procedures include biochemical, immunologic, and cell culture methods; in vitro cell-free and membrane-dependent systems; large and small laboratory animal models; and											
studies of human subjects.											
25. (U) 76 10 - 77 09 The role of altered pyridoxine metabolism in protection against											
malaria was examined in a field study in Ibadan, Nigeria, and in a comparative study of											
animals with varying susceptibilities to malaria. Nucleotide and glucose metabolism											
were examined in erythrocytes of P.knowlesi - infected rhesus monkeys, monkeys admin-											
istered prophylactic doses of chloroquine, and infected monkeys treated with chloro-											
quine. Alterations in heme synthesis in pyridoxine deficient animals were studied, and the defect localized to a single enzyme. The effect of removal of sialic acid from											
Itactor VIII on its activity was examined. Sialvi transferace activity was examined in											
Didtelets of Datients with hypercoagulability. Hemostatic alternations in mate with											
II. rhodeslense intection were examined. Proliminary studios wore done examining the											
fore of scress and exercise of fibrin cross linking Radioimmunoaccave for human co and											
provided of its fragments were established a novel mothod of protoin labeline wains											
tritiated borohydride, was developed. The role of lectins in modulating erythroid cell											
proliferation was examined. Lung cell culture systems were developed, and are now being used to study toxicity of inhaled noxious agents. For Technical Report see Walter Reed											
Army Institute of Research Annual Progress Report 1 July 76 - 30 September 77.											
				-por c	. outy	0 - 30	sep cellib	er //.			
			C	20							

PROJECT 3M161102BS01 RESEARCH ON MILITARY DISEASES

TASK 00 Internal Medicine

Work Unit 140 Military Hematology

Investigators

Principal: Michael J. Haut

Associate: David J. Ahr (until 9/76), Charles F. Barr, Jeffrey L. Berenberg (until 9/76), David H. Boldt (until 8/76), Bruce W. Booth, Carlos C. Daughaday, Richard C. Dimond, John A. Kark, Robert G. Knodell (until 8/76), Patrick E. Lorenz, August J. Salvado, Robert C. Smallridge, Stephen F. Speckart (until 8/77), Michael S. Steinberg (until 7/76), Edward A. Swabb, Harold L. Williams

### Description

Basic and clinical studies were performed to investigate the functions of blood and blood-forming organs. These focused on three specific areas: (1) study of normal blood cell metabolism and its alteration by disease or by toxic agents, (2) examination of blood cell surface membrane composition and function in normal and disease states, and (3) investigation of the mechanisms by which hemostasis is altered by disease, injury, physical stress, or medications.

#### Progress

1. Metabolism of normal blood cells and its alteration by disease or by toxic agents

The major emphases of our metabolic studies during the past year have continued to be on examining alterations in the metabolism of blood cells and certain other host tissues produced by infecting agents (bacterial or parasitic) or by toxic agents such as medications or environmental pollutants. Our studies on pollutant-induced alterations in metabolism of blood and bone marrow cells have grown in scope to the point that a new work unit for FY 78 has been established for that area. The particular metabolic areas we focused on were (a) heme synthesis by liver cell homogenates, reticuloycte lysates, and lysates of bone marrow erythroid precursors; (b) pyridoxine metabolism by erythrocytes; (c) purine nucleotide metabolism in erythrocytes; (d) red cell glucose catabolism; (e) heavy metal detection and toxicity;

and (f) oxygen toxicity and the role of superoxide dismutase.

### a. Heme synthesis

The major effort in this area has focused on investigation of the toxic effects of munitions-related pollutants, particularly the substituted benzenes and toluenes produced during manufacture of TNT. The effect of a number of these compounds on delta-aminolevulinic acid synthetase (ALAS) and ferrochelase (FC) activities in rat liver homogenates were determined, and attempts were made to derive structure-activity relationships from the data. these studies, the final concentration of each compound added to the reaction mixture was 10 mol/1. ALAS was measured by a modification of the method of Ebert, et al., (Biochem. Biophys. Acta 208:236:1970). FC was measured by a modification of the method of Bonkowsky, et al., (J. Clin. Invest. 56:1139:1975). Normal values for rat liver ALAS were 9-11 nmol/g protein and for FC 300-420 umol/g protein. Benzene and toluene showed a slight enhancement of ALAS activity but FC activity was depressed up to 25%. Ortho and meta-dinitrobenzene enhanced both ALAS and FC activities slightly but the para isomer suppressed these activities by 33-35%. This same trend was observed in the mono-chlorotoluenes. Para-nitrotoluene inhibited ALAS activity slightly but produced a 50% reduction in FC activity. Many diand trinitrotoluenes caused a greater than two-fold increase in FC activity. An amino group on the same ring with a nitro group appeared to negate the effect of the nitro group on FC activity. It appears from this preliminary data that orthoand meta-dinitrobenzene and poly-nitrotoluenes stimulate FC activity due to their steric properties while the para isomers inhibit these enzymes.

A second area of effort in the area of heme synthesis is examination of activities of ALA synthetase and ferrochelatase in bone marrow erythroid precursors of dogs rendered pyridoxine deficient. Previous studies in our department had shown an erythroid proliferative defect in the bone marrow of B6 deprived animals. This was demonstrated by plasma clot culture of bone marrow aspirates from dogs and monkeys rendered nutritionally defient in pyridoxine. The proliferative defect was not corrected by in vitro addition of erythropoietin to the culture system, but was corrected quantitativity by addition of pyridoxine to a rate of proliferation far above that of control animals. Becuase pyridoxine is known to be a cofactor for ALA synthetase and is thought to be important for ferrochelatase function, activities of these enzymes were examined in bone marrows of

dogs rendered nutritionally deficient in pyridoxine. Erythroblast ALA synthetase was found to be disminished to 50% of control levels; activity could be restored to normal by in vitro addition of pyridoxine to the marrow lysate. On the other hand, ferrochelatase activity reamined unchanged. The above results suggest that alterations in ferrochelatase activity which have been reported in patients with sideroblastic anemia are due to mitochondrial damage produced by accumulated iron rather than to alteration in availability of pyridoxine.

### b. Pyridoxine metabolism

The major effort in pyridoxine metabolism during the past year has been investigation of the role of pyridoxine metabolism in malaria. The majority of the these studies have been in collaboration with Drs. Louis Miller and Samuel Martin at NIH, Dr David Haynes from Division of CD&I, WRAIR, and Drs Esan and Osunkoya and their coworkers at the University College Hospital, Ibadan, Nigeria.

The observation that American blacks have decreased pyridoxal kinase (PLK) activity (Science 187:1084, 1975) raised the possibility that low PLK increases survival in regions holoendemic for falciparum malaria. To evaluate this hypothesis, PLK activity was determined in hemolysates from Nigerian children 4 months old to 5 years old, five weeks after successful treatment of severe falciparum malaria (1% to 20% parasitemia), and from uninfected matched controls. Science both PLK and glucose-6-phosphate dehydrogenase (G6PD) decrease as red cells age, G6PD was measured as a marker for red cell age. PLK in 24 uninfected controls was lower than in 19 patients (p<0.001). No correlation was found between PLK and G6PD; hence it is unlikely that this difference in PLK was a function of red cell age. The data suggested that decreased erythrocyte PLK may protect against development of severe falciparum malaria infections. pursue these studies further, a number of studies have been initiated: (1) In collaboration with Dr Martin, erythrocytes of thirteen animal species have been examined for PLK activ-In twelve of the thirteen species, presence of PLK correlated with ability to be infected by malarial parasites, and absence with inability to be infected. (2) In collaboration with our group, Dr David Haynes has cultured P. falciparum in human erythrocytes in a special medium which contained no pyridoxine other than the miniscule amount bound to serum. A number of inhibitors have been selected for testing, in this system, and arrangments are being made to obtain these. (3) More detailed study of the variants of

pyridoxal kinase are currently underway. Theses include detailed studies of kinetic parameters of the enzyme, examination of inhibition of the various forms of the enzyme by known inhibitors, and studies of stability of the different forms of the enzyme, using cells separated into various age ranges by centrifugation on a Stractan gradient.

In addition to the malaria studies, pyridoxine-related studies have focused on the role of pyridoxine in heme biosynthesis and erythropoiesis, the effect of isoniazid (INH) on pyridoxine metabolism, and the metabolic effects of pyridoxine administration to patients with sideroblastic anemias. Progress in the first of these areas has been described above (under 1.a. Heme biosynthesis). Progress in the other two areas will be discussed below.

Previous studies in our department demonstrated that patients taking 300 mg daily for prophylaxis of tuberculosis had lower erythrocyte pyridoxal-5-phosphate (PLP) levels and pyridoxal kinase (PLK) levels than matched control subjects. Experiments were undertaken during the past year to confirm these results and to determine the mechanism of inhibition. To avoid the effect of individual variation, PLK activity was determined in 14 subjects prior to initiation of INH prophylaxis, and at various intervals after the drug had been started. PLK decreased in 13/14 tuberculin converters after taking INH for 1 to 3 months (mean + SEM, from 389 + 36 to 302 + 30 p m PLP/g hgb/min, p<0.001, but no change</p> occurred  $\overline{\text{in}}$  15 control subjects (from 355 + 23 to 365 + 23). Since we were able to show that INH did not inhibit PLK in vitro, we focused our attention on pyridoxal isonicotinylhydrazone (PL-INH), a natural metabolite of INH which has been shown to inhibit brain ( $K_T = 4.8 \mu M$ ) and liver PLK in This compound was synthesized, its purity established by several methods (TLC, GS-mass spectrometry, NMR, infrared spectroscopy), and its effect on erythrocyte PLK was determined. Addition of PL-INH to hemolysates caused noncompetitive inhibition of PLK at levels above 1  $\mu M$  (K<sub>T</sub> = 1.5 and 1.6  $\mu M$  PLINH) and uncompetitive inhibition at lower levels (0.5 to 0.8  $\mu M$  PLINH). PL-INH was a noncompetitive inhibitor of red cell PLK in vitro at levels low enough to suggest that this may account for the in vivo decrease in red cell PLK when patients take INH.

Metabolic studies of erythrocytes from patients with sideroblastic anemia were completed. Plasma and RBC PLP levels and rates of RBC PLP synthesis from pyridoxal and pyridoxine (PN) were determined on paired blood samples from 19 patients with idiopathic refractory sideroblastic anemia (IRSA) and 20 racially matched healthy persons. Twelve

patients had not received PN for at least 6 months prior to study. Levels of plasma PLP in these patients were very low (mean  $\pm$  SD, 3.6  $\pm$  2 ng/ml vs normals, 10.1  $\pm$  4). RBC PLP levels were normal (0.69  $\pm$  0.5 ng/10 RBC vs 0.62  $\pm$  0.4). Rates of PLP synthesis were normal (8 patients) or elevated (4 patients). Ten patients were studied during treatment with PN, including the 3 patients with the lowest pretreatment RBC PLP levels and lowest rates of RBC PLP synthesis. Plasma PLP levels were low (4 patients) or moderately elevated (6 patients, 25-40 ng/ml). RBC PLP levels were markedly elevated (4.9 + 2 ng/10 RBC). Rates of PLP synthesis were normal (3 patients) or very high (2 x normal in 6 patients). The restudied patients showed a 7-fold increase in RBC PLP, a 2-fold increase in rates of RBC PLP synthesis, but only a mild inconsistant rise in plasma PLP. Thus all patients maintained adequate levels of RBC PLP and rates of RBC PLP synthesis before treatement. During PN treatment there was a substantial increase in levels of RBC PLP and a moderate increase in rates of RBC PLP synthesis. No hematologic response to PN occurred despite a good biochemical response in RBC's. This suggests that abnormalities of red cell vitamin B6 metabolism were not the primary cause of anemia in these 19 causes of IRSA.

#### c. Purine nucleotide metabolism

Changes in content and distribution of blood nucleotide were studied in rhesus monkeys under three conditions: synchronous infection with P. knowlesi, chloroquine (CQ) prophylaxis, and chloroquine treatment of fulminant malaria infection. This study is part of a continuing collaborative effort between CPT H. K. Webster (Division of Logistics), LTC M.J. Haut (Department of Hematology, Division of Medicine), MAJ L. Martin (Division of Communicable Diseases & Immunology), and LTC P. Hildebrandt (Division of Experimental Pathology). There were two hypotheses at issue. First, a cyclic variation in the levels of erythrocytic nucleotides may occur during the course of infection with P. knowlesi in the subject monkeys. This variation may bear a determinant influence on periodicity of parasitemia and be characteristic for the parasite's developmental asexual blood stages. Second, chloroquine may exert its chemotherapeutic action by depletion of erythrocytic ATP levels. Perchloric acid extracts were examined using a high performance liquid chromatographic system (HPLC), with a microparticulate anion exchange column. Dramatic changes were observed in adenylates, although variations were recorded for all major ribonucleotides. Infected monkeys followed over 2-3 cycles showed variations in nucleotide

pools characteristic of the predominant schizogonic growth stage. During "ring" state, ATP levels (ATP), total adenylates ( $\Sigma$ AXP) and adenylate energy charge (AEC) were maintained at or above basal values independent of parasitemia. During trophozoite stage and schizogony, the above parameters were markedly decreased. Uninfected monkeys given therapeutic doses of CQ exhibited dose/time dependent decreases in !ATPl and AEC whereas  $\Sigma$ AXP decreased slightly. Return toward basal levels correlated with the biological half-life for CQ in monkeys. In CQ treated monkeys, ATP levels decreased, AEC stabilized, and stage-related variations in these two parameters did not occur.

To investigate in more detail the relationships between erythrocyte and parasite metabolism, assays for the purine interconversion enzymes have been set up and standardized. Activity of each of these enzymes is currently being determined in uninfected erythrocytes, in isolated parasites, and in infected erythrocytes.

#### d. Glucose metabolism

Alterations in glucose metabolism in monkeys with a synchronous P. knowlesi infection have been examined at the same time as nucleotide metabolism in the animals described above (section l.c. Purine nucleotide metabolism). Measurement of glycolytic enzymes in erythrocytes from these monkeys reveal an increase in activity of pyruvate knase and hexokinase in all animals following inoculation with stabilate, usually beginning before parasitemia was patent. In some animals, glucose-6-phosphate dehydrogenase and phosphofructokinase were elevated as well. In collaboration with Dr Richard Carter from the Laboratory of Parasitic Diseases, NIH, we have set up starch gel electrophoresis for each of the above enzymes to determine whether they are host or parasite enzymes.

In collaboration with Drs M. Wood and A. Harken of the Division of Surgery, WRAIR, and Dr Maria Delavoria-Popadopoulos from the University of Pennsylvania School of Medicine, a method was developed for augmenting 2,3-DPG in dog erythrocytes. In red cells of most mammalian erythrocytes, 2,3-DPG can be elevated by incubation of stored blood with inosine, pyruvate, and inorganic phosphate. In the presence of the red cell enzyme purine nucleoside phosphorylase (PNP), inosine plus phosphate is broken down to ribose-1-phosphate and hypoxanthine. The ribose-1-phosphate is subsequently converted to 2,3-DPG within the red cell. Since the preferred laboratory animal for most forms of surgical research is the dog, and since the dog has been

demonstrated to have no erythrocyte PNP activity. (Blood 39:525, 1972) an attempt was made to find an alternative method of augmenting 2,3-DPG in this animal. Since neither dihydroxyacetone nor glyceraldehyde are charged and therefore enter the red blood cell with relative eass (Blood 41:559, 1973), where they are then phosphorylated by triokinase, dihydroxyacetone was chosen. Infusions containing dihydroxyacetone, along with pyruvate (to permit NAD regeneration by conversion of pyruvate to lactate, thereby allowing all 1,3-DPG formed from the dihydroxyacetone to proceed to 2,3-DPG) were administered to thirty dogs. The concentration of 2,3-DPG was initially 16.2+ 0.4 µM/gm Hb and increased to 18.6+ 0.6 µM/gm Hb (mean + SEM) in dogs infused with the dihydroxyacetone -pyruvate solution. Concurrently, the P50 increased from 27.6 + 0.9 torr to 31.76 + 0.6 torr in these animals. Triokinase was demonstrated in erythrocytes of all dogs. No changes were seen in either 2,3-DPG levels or P50 when animals were administered either saline or pyruvate alone.

### e. Heavy metal detection and toxicity

A method was developed for simultaneous measurement of iron and copper ions in 0.2 ml of serum after using guanidine hydrochloride to cause their release from protein. Sensitive reagents for iron (2,4,6-tripyridyl-5-triazine) and copper (disodium 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline disulfonate) were used to determine the level of the freed metal ions. Spectral interference between the two metal-ion complexes was eliminated by selective use of pH. Results of the method compared well with those obtained by accepted methods in which the concentration of these ions are measured independently.

Similarly, a method was developed for the improved colorimetric determination of serum zinc. This method also uses the principle of protein perturbation without precipitation to effect quick release of protein-bound metal.

In collaboration with Dr D. Hawkins (Division of Neuro-psychiatry), a newborn rat model has been developed for quantitative assessment of lead encephalopathy and response to treatment. Lead is administered to newborn rats crossbred to be (a) drinker rats, which will take the lead-containing water and (b) auditory seizure-sensitive for quantitation of siezure thresholds by sound stimulation. This model will be used to examine the potential usefulness of pyridoxine in treating lead encephalopathy. Lead has been shown to inhibit pyridoxal kinase, and intramuscular pyridoxal phosphate has been shown to rapidly reverse peripheral neuro-

pathy due to lead poisoning.

### f. Oxygen toxicity and the role of superoxide dismutase

In collaboration with Maj A. Harken and SP5 R. Lillo of the Division of Surgery, WRAIR, the effect of oxygen tension (Po,) on oxygen consumption (Vo,) of rabbit liver slices was investigated to determine the relationship between extracellular fluid Po, and liver slice Vo,. Seventy rabbits, 5 kg each, were sacrificed with air embolism. Liver slices (0.3 gm) in Krebs-Ringer's-phosphate (KRP) dextrose were placed in a constantly agitated, 37°C, closed cuvette, and Po 2 was continuously monitored down to 10 torr. The system was reoxygenated and closed, and observations were repeated. Hemoglobin concentration was measured on the liver slice homogenate, and a  $P_{50}$  was measured on autologous blood. The presence of small amounts of hemoglobin in the supernatant was confirmed by electrophoretic, spectrophotometric, and morphologic studies. The pH decreased from mean 7.47 to mean 7.37 during each run. The resultant increase in  $P_{50}$  was recalculated and included in the determination of each  $\Delta 0_2$  content/min (Vo<sub>2</sub>). Vo<sub>2</sub> at Po<sub>2</sub> 30 torr was greater than Vo<sub>2</sub> at Po<sub>2</sub> 90 torr or 10 torr (p<0.0001 in each case). Vo<sub>2</sub> at Po<sub>2</sub> 30 torr was not significantly different in the first vs the second run (p>0.85). The critical oxygen tension for hepatocyte respiration appears to be 30 torr.

Studies examining oxidant injury in the lung have also been initiated. Work in this area so far has been concentrated on the isolation and culture of Type II pneumocytes and establishment of assays. Methods have been set up to measure superoxide dismutase activity by two methods: (a) lipid peroxidation by both malondialdehyde release and conjugated diene fluorimetry, and (b) superoxide generation by cytochrome c reduction and chemiluminescence. Application of these assays to an in vitro oxygen toxicity model is in progress.

### 2. Examination of blood cell surface membrane composition and function in normal and disease states

Our department's studies of the role of blood cell surface membrane composition and function have been extended this past year to include membranes of several types of cells in addition to the lymphocytes. In particular, studies are in progress which involve erythroid precursor cells, platelets, and mononuclear phagocytes.

### a. Erythroid precursor cells

In vitro growth of erythroid stem cells in plasma clot cultures is being used to elucidate the role of the cell surface in regulating stem cell proliferation, and the interactions between lymphocytes and stem cell growth.

Lectins have been used to look at the role of the cell surface in stem cell proliferation. The three lectins used have known receptors on mature red cells; Concanavalin A, Wheat Germ Agglutinin, and Phytohemagglutinin. Initially each of the lectins was added in varying doses to standard plasma clot cultures using murine marrow. Dose response curves were generated for each lectin and it was found that all three lectins had significant effects on stem cell growth in extremely low doses. Two lectins (WGA and PHA) suppressed stem cell growth while the third (Con A) stimulated growth. Since WGA and PHA have been noted by others to bind erythropoietin which is necessary for erythroid stem cell growth, we used preincubation studies as well as binding studies with 125 I labelled lectin to determine whether this accounted for the suppression noted. We found in fact that the lectin aside from binding to erythropoietin have a direct effect upon some cell present within the marrow population. That cell could either be the stem cell itself or an intermediate cell such as a lymphocyte. The importance of the possible generation of "Suppressor" lymphocytes by these agents lies in the fact that within the past year several clinical investigators have documented that a significant number of patients with severe bone marrow failure of unknown etiology have been found to have "suppressor" lymphocytes in both their peripheral blood and bone marrow. These patients are potentially capable of improving their marrow function when treated with antithymocyte globulin to eliminate the pathologic clone of lymphocytes. Our system then might serve as an excellent model to look at the mechanism for lymphocyte suppression of stem cell growth and to look for other important exogenous agents capable of producing bone marrow failure by similar mechanisms.

Investigations presently being undertaken by us then include: (a) co-culture experiments in which lectin stimulated purified murine lymphocytes are cultured with normal murine marrow to look for suppression of stem cell growth, and (b) purification of erythroid stem cells by unit gravity velocity sedimentation followed by determination of certain normal molecular parameters such as cyclic AMP and nuclear phosphorylation after erythropoietin production and investigation of alterations in the normal sequence by previous alteration of the stem cell by lectins and/or other agents.

#### b. Platelets

Increased platelet surface sialytransferase activity (PSSA) has been shown to be associated with epinephrine and ADP induced platelet aggregation (Wu K. K. et. al., Thrombos. Haemostas 38:8, 1977). As part of our department's effort to examine in detail the hypercoagulable state, the relationship between enhanced platelet aggregation, hypercoagulability and platelet surface sialytransferase activity in a group of patient's with cancer-associated hypercoagulability has been examined. Coagulation parameters, factor VIII antigen (VIII Ag), ristocetin cofactor activity (VIII VWF), platelet sialic acid, platelet aggregation, and PSSA were examined in these patients. PSSA was measured by the ability of intact platelets to incorporate C -CMP sialic acid into exogenous (asialofetuin acceptors) and endogenous (platelet membrane) receptors (modified from Boseman H.B. Biochem. Biophys. Acta. 279: 459,1972). The cancer patients demonstrated enhanced platelet aggregation, increased VIII Ag and VIII VWF activity, and increased exogenous PSSA as manifestations of hypercoagulability. Of 12 cancer patients, 6 had major thrombotic episodes (pulmonary embolus - 2, stroke -2, deep venous thrombosis - 1, and acute hepatic artery thrombosis -1). This data suggests an association between increased PSSA, ehanced platelet aggregation, hypercoagulability and thrombosis in these patients.

### c. Mononuclear phagocytes

Investigations of membrane functions of mononuclear phagocytes have focused on alevolar macrophage function in respiratory infection, and on monocyte interactions with Dengue Fever virus.

Antibody dependent cellular cytotoxicity assays have been adapted for use with pulmonary alveolar macrophages. Bronchus associated lymphoid tissue (BALT) cells have been separated from the lungs of guinea pigs, and work has begun on characterizing their function in cytotoxicity assays. In vitro modulation of macrophage function by the pharmacologic agents levamisole and polynucleotides is being examined. These agents appear to enhance cytotoxic activity of both pulmonary alveolar macrophages and BALT cells.

F(ab')2 fragments from specific dengue immune serum has been prepared. This will be tested by Dr Walter Brandt, Division of Communicable Diseases and Immunology for dengue virus neutralization. If the fragments retain neutralization activity, they will be used as a tool to study the role of Fc receptors in the immune serum-enhanced viral replication

in monocytes.

### d. Lymphocytes

Work was carried to completion on several studies of lymphocyte membrane structure and function. Since preliminary results for these studies were described in last year's annual report, only a brief description of each of these will be presented.

Binding studies with six purified plant lectins were used to investigate membrane alterations that occur in lymphocyte transformation. Normal human peripheral blood lymphocyte transformed with E-PHA or con-A generally possessed increased numbers of lectin receptors. When this increase was corrected for the expanded surface area of transformed lymphocytes, it appeared that E-PHA and con-A each produced a unique and complex reorganization of cell surface to pography. Surface alterations occurred independently of DNA synthesis, cell proliferation, and microtubule or microfilament function. Puromycin inhibited emergence of new lectin receptors on cells transformed with E-PHA, but not with con-A. Lymphocytes incubated with either lectin showed increased incorporation of <sup>14</sup>C-galactose into trypsinsensitive cell surface glycoprotein. This incorporation was abolished by puromycin in cells stimulated by E-PHA but not by con-A. These studies demonstrate that although both lectins induce similar morphological alterations in human lymphocytes, at the molecular level the structural changes induced in the cell membrane by these two lectins differ considerably. Furthermore, these structural alterations are mediated via different mechanisms in the two groups of De novo protein synthesis is required for cell surface reorganization in phytohemagglutinin-stimulated cells, but not in cells stimulated by concanavalin A. The effect of con-A appears to be to enhance attachment of saccharide structures to presynthesized membrane proteins.

A large proportion of DNA synthesized in vitro by human lymphocytes stimulated with plant mitogens or specific antigens is selectively excreted from the cells. To determine if DNA excretion differs among various types of lymphocytes, we examined purified human lymphocyte subpopulations for DNA synthesis and excretion in response to stimulation by L-PHA. The relative proportion of newly synthesized DNA that is excreted by unseparated mononuclear cells, macrophage-depleted cells, T, and B lymphocytes is identical despite great differences in the magnitude of their responses. Low levels of both DNA synthesis and

excretion by macrophage-depleted cells and B cells can be increased by reconstitution with macrophages and T cells, respectively. These data indicate that DNA excretion is a general property of lymphocytes stimulated to undergo DNA synthesis by plant mitogens.

The ability of the mitogenic components of phytohemagglutinin (PHA) and the nonmitogenic lectin, wheat germ agglutinin (WGA) to cause autologous cellular cytotoxicity of human red blood cells were compared. E-PHA demonstrated gradually increasing effectiveness in the induction of autologous cellular cytotoxicity over a concentration range of 4.2 to 83 µg/ml. L-PHA did not induce any cytotoxicity over a range of 5 to 270 µg/ml. By contrast, WGA was effective at lower concentrations than E-PHA in inducing cellular cytotoxicity and exhibited a maximum effect over a narrow range between 4 and 8 µq/ml decreasing in potency at higher concentrations. Both E-PHA and WGA produced their peak cytotoxic effect at killer-to-target cell ratios of approximately 25:1. In addition, WGA was capable of inducing cytotoxicity (37.8%) at a lower killer-to-target cell ratio (1:1) than was E-PHA.

We investigated mechanisms by which plant lectins induce human peripheral blood mononuclear cells to kill red blood cells (RBC) from different species selectively. Cytotoxicity was induced by both mitogenic components of phytohemagglutinin-P (PHA), erythroagglutinating (E-PHA) and leukoagglutinating (L-PHA), and the nonmitogenic lectin wheat germ agglutinin (WGA). The target cells used in an overnight chromium release assay included human autologous RBC, human allogeneic RBC, and xenogeneic RBC from sheep or chickens. Although E-PHA induced cytotoxicity for all cell types, L-PHA caused human mononuclear cells to kill only xenogenic RBC and conversely, WGA induced killing of only human RBC. These differences allowed further investigation of possible control mechanisms. The target cell specificity associated with lectin-induced cellular cytotoxicity did not correlate with lectin binding to or agglutination of, the different red blood cell types. Furthermore, preincubation of RBC with lectins followed by washing did not result in cytotoxicity. However, when the mononuclear cells were preincubated with the lectins, the same cytotoxic specificity was observed as when lectins were present during the entire assay. These experiments suggest that the target cell specificity observed with lectin-induced cellular cytotoxicity is related to a prearmed lymphocyte which seeks out and kills the appropriate target cell.

The development of profound leukocytosis in a patient with

leukemic reticuloendotheliasis (LRE) enabled us to obtain purified LRE cells for the investigation of their structural and functional characteristics. The LRE cells of our patients bore surface immunoglobulin and had complement receptors but did not bear F receptors and did not form rosettes with sheep erythrocytes. By electron microscopy, the cells were observed to contain typical ribosome lamella structures and to phagocytize both 0.81 µm latex particles and complement-coated zymosan particles. They were adherent to both glass and nylon wool fibers. The mitogenic response to both grass and hyron most to erythroagglutinating phytohemagglutinin was normal in to erythroagglutinating phytohemagglutinin was normal in the binding of magnitude but delayed chronologically. The binding of labeled plant lectins was used to characterize the surface topography of LRE cells. Results of these studies indicated that the LRE cell surface differed significantly from the surface of normal T and B lymphocytes and chronic lymphatic leukemia cells. The LRE cells were capable of both stimulating and responding in a one-way mixed lymphocyte culture. However, the LRE cells were not active as effector cells of either cell-mediated lympholysis, a T cell function, or antibody-dependent cellular cytotoxicity, a null cell function. In contrast, they were effector cells of lectininduced cellular cytotoxicity showing that they did possess the capacity to function as cytotoxic effector cells. These data indicated that the LRE cells in our patient had surface and functional charcteristics of both lymphocytes and monocytes.

A panel of five plant lectins with different binding specificities was used to determine if plant lectins could bind specifically to membrane-associated glycolipids. Ricinis communis and wheat germ agglutinins both bound specifically to mixed brain gangliosides and globoside I from human erythrocytes. Wheat germ agglutinin also bound to ganglioside  $G_{MI}$  and human erythrocyte ceramide trihexoside, but not to ceramide dihexoside, mono-, or digalactosyl diglycerides. Concanavalin a bound to liposomes with or without glycolipid subststituents, and this binding was partially inhibited by  $\alpha$ -methyl mannoside. This study indicates that lectins can specifically recognize and bind to certain glycolipids in membranes.

# 3. Alterations in hemostasis produced by disease, injury, physical stress, or medications

A major effort of our hemostasis studies during the past year has been to examine biochemical mechanisms underlying hemostatic alterations in several disease states. These studies include: (a) examination of the effect of neuraminidase treatment of factor VIII/von Willebrand factor on its functional integrity, (b) development of a radioimmunoassay for fragments of the fibrinogen alpha chain, (c) development of a radioimmunoassay for the third component of complement, and (d) completion of studies examining effects of endotoxin on tissue factor generation by human mononuclear cells. In addition, studies have been completed describing (e) hemostatic alterations in rats infected with Trypanosoma rhodesiense, (f) the clinical and laboratory findings in a patient with uncontrolled gastrointestinal bleeding secondary to combined hemostatic defects (von Willebrand's disease and hemorrhagic telangiectasia, (g) acquired platelet dysfunction following mithramycin therapy, and (h) hemostatic profiles of patients with various clinical manifestations of hypercoagulability.

# a. Reduced ristocetin-induced aggregation of normal humor platelets after neuraminidase treatment of factor VIII/von Willebrand factor

Normal human factor VIII/von Willebrand factor (f VIII/vWf) was purified by gel filtration, followed by treatment with protease-free Vibrio Cholera neuraminidase (VCN) to release 70% of its sialic acid content. The desialized f VIII/vWf was compared to untreated f VIII/vWf for both procoagulant activity and platelet aggregation in a standard ristocetin-induced platelet aggregation (RIPA) system. The neuraminidase treated f VIII/vWf contained normal proagulant activity but demonstrated a 78% reduction in ristocetin cofactor activity. To examine more completely the chemical interaction between VCN and f VIII/vWf, large volumes of both neuraminidase and f VIII/vWf were incubated together using identical conditions as mentioned for the procoagulant and ristocetin studies. After incubation this mixture was dialyzed, concentrated, then eluted over a Sephadex G-150 column. 25% of the free sialic acid was recoverable in the dialysate and column chromatography revealed complete dissociation of the two protein peaks indicating that the interaction of VCN on f VIII/vWf was by desialization only. Carbohydrate analysis of the purified f VIII/VWF demonstrated that 5.2% of this protein, by weight, contained sugar residues which include 0.98% sialic acid, 1.64% hexose, and 2.55% hexosamine. These data indicate that human f VIII/vWf is a glycoprotein containing 1% sialic The presence of this sugar residue on f VIII/vWf is not necessary for procoagulant activity. However, the absence of sialic acid significantly inhibits the ability of f VIII/vWf to effect platelet aggregation in the ristocetin assay.

# b. Development of a radioimmunoassay for fragments of the fibrinogen alpha chain

In collaboration with Dr. Earl Ferguson, USUHS, we are in the process of developing a radioimmunoassay for peptide fragments of the fibrinogen alpha chain that contain the acceptor site for crosslinking of fibrinogen related peptides involved in fibrinogenolysis, formation of fibrin monomer, and fibrinolysis. We have recently received enough purified protein from Dr. Ferguson to initiate these studies.

Antisera to the alpha chain peptides are being raised in New Zealand white rabbits and are being characterized for specificity and sensitivity. To date, we have obtained four antisera. In addition, methodology is being developed for the radioiodination of these peptides and their characterization by gel chromatography and SDS polyacrylamide gel electrophoresis.

### c. Development of a radioimmunoassay for the third component of complement

In collaboration with Dr Brian Tack (American National Red Cross) and Dr Alan Schechter (Laboratory of Chemical Biology, NIAMDD, NIH), Cpt Lorenz has developed a radioimmunoassay for the third component of human complement. The third component of human complement (C3) was purified to homogeneity by the method of Tack and Prahl (Biochem. 15: 4513, 1976). Sheep and rabbits were made hyperimmune with C3, as well as with activation fragements C3b, C3c, and C3d, as determined by standard double-diffusion assays. Radiolabeling of C3 and each of the fragments was performed by reductive alkylation with tritiated sodium borohydride (10 Ci/mmol) and formaldehye. This method, which effects the methylation of free aminogroups, allowed labeling to specific activities of ~ 4 x 10 cpm/μg, with minimal change in function as determined by the hemolytic activity assay for C3. Competitive binding studies were conducted by incubation of the labeled and native C3 proteins with a 1/10,000 dilution of the sheep antiserum and free protein was separated from bound with a burro antisheep IgG antiserum. This radioimmunoassay indicted an affinity constant of about 5 x 10 liters/mol and with 100  $\lambda$ samples could be used quantitatively in the range from 2 to 80 ng of C3. The affinities of native and labeled C3 for the antibodies were equivalent. Direct radioimmunoassay and quantitative inhibition studies with each of the fragments are in progress to evaluate the antigenic relationships among these proteins with the goal of developing specific

assays for each of these activation fragements. However, the present C3 assay itself should have utility in clinical studies and in vitro measurements of the complement pathway.

# d. Effects of endotoxin on tissue factor generation by human mononuclear cells

The effects of the presence of endotoxin in a mononuclear cell culture system were assessed. Endotoxin was shown to be mitogenic for human peripheral blood lymphocytes and capable of stimulating the generation of tissue factor. Concentrations of endotoxin, found to contaminate many commercial mitogens and antigens, activated mononuclear cells in a time-dependent manner. Generation of tissue factor was detected in cultures harvested from 2 to 72 hours following stimulation with endotxin. Dose-response curves relating concentrations of endotoxin to mononuclear cell stimulation were determined; as little as 0.0001 µg/ml of E coli endotoxin was capable of stimulating the generation of tissue factor in the cell cultures. The mitogenic effect of endotoxin was modest, however, and appeared to be unrelated to the ability of endotoxin to activate tissue factor. Inhibition of DNA synthesis in the cell cultures by cytosine arabinoside or nonlethal irradiation failed to impair the generation of tissue factor. Endotoxin contamination of various reagents used in cell culture was evaluated with the Limulus assay, which detected as littled as 1 x 10 of endotoxin. Endotoxin was detected in preparations of phytohemagglutinin, purified protein derivative of the tubercule bacillus, mumps vaccine, tetanus toxoid, concanavalin A, and pokeweed mitogen. Because of the broad implications of contamination by endotoxin of various reagents, we assessed the specificity of the Limulus assay for the detection of endotoxin in the lectin, concanavalin A, and determined that the reaction was specific for endotoxin. Contamination by endotoxin of mononuclear cell culture systems should be considered as a possible factor in the production of various biological effects attributed to some commonly used mitogens and antigens.

# e. Hemostatic alterations in rats infected with Trypanosoma rhodesiense

Rats were infected with Trypanosoma rhodesiense (EATRO 1886) and groups of animals were exsanguinated at weekly intervals for four weeks. Platelet counts, plasma fibrinogen concentrations, prothrombin times, C3, C4, whole complement levels, and alternative pathway activation were monitored. Routine hematologic tests were performed and

tissues were examined histologically.

A 37% reduction in platelets was noted one week after inoculation with a progressive reduction of up to 94% by the fourth week. In spite of these marked changes in platelet counts, no systematic abnormalities in the other parameters of coagulation were observed. A drastic decrease in C4 was observed beginning two weeks after infection and continued through the fourth week. Beginning three weeks after infection, C3 levels decreased markedly and there was evidence of alternative pathway activation. Whole complement levels decreased from 11,520 CH<sub>50</sub> units/ml prior to inoculation to less than 320 CH<sub>50</sub> units ml 3 weeks after infection. Anemia was also evident in the animals. At four weeks post-infection there was a 50% decrease in erythrocyte counts with accompanying decreases in hematocrit and hemoglobin levels. The mean corpuscular volume increased by 28%, and the final blood samples revealed a high percentage of nucleated erythrocytes indicating regeneration of the erythron. A brisk leukocytosis was also observed. Histologic evidence of glomerulonephritis was evident in infected animals.

The lack of evidence of coagulation factor activation suggests that, in this model, thrombocytopenia may proceed through pathways not involving DIC. Platelet destruction through mechanisms involving immune complexes constitutes one reasonable hypothesis to explain the observed phenomena.

### f. von Willebrand's disease and hemorrhagic telangiectasia

The clinical and laboratory findings in a patient with uncontrolled gastrointestinal bleeding secondary to combined hemostatic defects (von Willebrand's disease and hemorrhagic telangiectasia) are described. Evidence for von Willebrand's disease was found in five family members, but no other affected relative was found to have hemorrhagic telangiectasia. Complete assessment of the patient and her family included determination of factor VIII activity, factor VIII antigen and von Willebrand factor levels. The patient described also was evaluated for her response to transfusion utilizing these same measurements.

# g. Acquired platelet dysfunction following mithramycin therapy

Bleeding time, platelet aggregation, platelet nucleotide levels, and coagulation factor assays were

studied in three patients receiving mithramycin for embryonal testicular carcinomas. These studies demonstrated a cyclic, reversible hemorrhagic diathesis associated with (1) prolongation of the bleeding time, (2) decreased platelet aggregation responses to ADP, collagen, and epinephrine, and (3) depleted platelet stores of ADP in the absence of thrombocytopenia. These abnormalities were temporally correlated with the onset of mucocutaneous bleeding in all patients.

### h. Hemostatic profiles of patients with various clinical manifestations of hypercoagulability

Hypercoagulability is a serious complication of a number of disorders that might be encountered in a military situation. Patients with trauma, burns or exposure to extreme environments (heatstroke, cold exposure, hyperbaric pressure) are particularly prove to develop this complication. Since it is extremely difficult to obtain specimens from patients with the above medical problems, we have examined two other groups of patients known to have frequent thrombotic problems-patients with severe vascular disease and patients with certain types of malignancy. Techniques and concepts developed in our studies of these two groups will later be applied to patients with hypercoagulability due to trauma, burns, or environmental exposures.

During a one and a half year period, detailed hemostatic data were obtained on forty five patients with clinical suspicion of hyperocagulability based on well documented thrombotic episodes. Seventeen of these patients had purely arterial, fourteen pureley venous, and fourteen combined arteriovenous thrombotic disease. By eliminating tests with low yields and those duplicating information from some other test, a condensed coagulation profile consisting of six tests was derived: fibrinogen (56% abonormal); factor VIII (36%); factor XI (38%); activated PTT (46%); progressive antithrombin activity (20%); epinephrine-induced platelet aggregation (64%). The condensed coagulation profile agreed with the clinical profile in 91 per cent of patients and disagreed with the clinical profile in 9 per cent of patients. In three patients (7 percent) the clinical impression of hypercoagulability was quite strong and the test results were considered false-negative; in one patient ( 2 per cent) the laboratory profile was positive but the clinical profile had been overemphasized, accounting for false-positive results.

Coagulation and platelet function studies were evaluated in eighteen patients with colon cancer (all clinical stages)

prior to treatment with chemotherapy. All patients were asymptomatic from bleeding or thrombosis and were not taking aspirin. The tests included general coagulation screening, coagulation factor analyses, platelet aggregation, fibrin split product concentration and a serial dilution protamine sulfate test for the presence of fibrin monomer. Average values compared to normal controls showed an elevated fibrinogen 400.5 mg% (normal 300.0 mg%), shorter activated partial thromboplastin time 29.4 seconds (normal 36.0), increased factor VIII coagulant activity 127.2% (normal 110%), and elevated fibrin split products 28.8 µg/ml (normal less than 16  $\mu$ g/ml). Seven patients showed the presence of fibrin monomer. Eight patients showed enhanced aggregation reacting at or below 0.16 micromolar epinephrine. Six patients in the study showed more advanced cancer (five requiring intrahepatic artery infusion for extensive liver metastasis). Of this subgroup four patients demonstrated fibrin monomer and three patients showed spontaneous platelet aggregation. In this group, the fibrinogen was 484.2 mg%, fibrin split products were 28.8  $\mu g/ml$ , and factor VIII was 176%. A state of hypercoagulation with signs of a chronic process of intravascular coagulation was demonstrated in a group of colon cancer patients which corresponded to their extent of disease.

PROJECT 3M161102BS01 RESEARCH ON MILITARY DISEASES

Task 00 Internal Medicine

Work Unit 140 Military Hematology

### Literature Cited.

#### Publications:

- 1. Ahr, D.J., Rickles, F.R., Hoyer, L.W., O'Leary, D.S. and Conrad, M.E. von Willebrand's disease and hemorrhagic telangiectasia: Association of two complex disorders of hemostasis resulting in life-threatening hemorrhage. Am. J. Medicine 62:452-458, 1977
- 2. Boldt, D.H., Mac Dermott, R.P., Speckart, S.F. and Nash, G.S. Excretion of DNA by purified human lymphocyte subpopulations. J. Immunology <u>118</u>:1495-1498, 1977
- 3. Boldt, D.H., Nash, G.S., Speckart, S.F. and Mac Dermott, R.P. Cellular cooperation in the release of DNA by human lymphocytes. Proceedings of the Eleventh Leukocyte Culture Conference, 1976
- 4. Boldt, D.H., Speckart, S.F., Mac Dermott, R.P., Nash, G.S. and Valeski, J.E. Leukemic reticuloendotheliosis: "Hairy cell leukemia", functional and structural features of the abnormal cell in a patient with profound leukocytosis. Blood 49:745-757, 1977
- 5. Boldt, D.H., Speckart, S.F., Richards, R.L. and Alving, C.R. Interactions of plant lectins with glycolipids in liposomes. Biochem. Biophys. Res. Comm. 74:208-214, 1977
- 6. Collins, G.J., Ahr, D.J., Rich, N.M. and Anderson, C.A. Detection and management of hypercoagulability. Am. J. Surgery 132:767-770, 1976
- 7. Collins, G.J., Rich, N.M., Scialla, S.J., Anderson, C.A. and McDonald, P.T. Pitfalls in peripheral vascular surgery: Disseminated intravascular coagulation. Am. J. Surgery 134:375-380, 1977
- 8. Daughaday, C.C. Superoxide anion: A mediator of free radical injury in the lung. (Presented at the 30th Annual Symposium on Pulmonary Diseases held at Fitzsimmons Army Medical Center, Sep 77).

- 9. Ferguson, E.W. and Barr, C.F. Effects of exercise on blood coagulation, fibrinogen, factor assays, platelet function, and fibrin-cross linking. (Submitted to Society of Air Force Physicians, Dec 77).
- 10. Flemmings, B., Barr, C., Toussaint, A., Johnson, A., Schneider, I., Haut, M. and Diggs, C. Thrombocytopenia without disseminated intravascular coagulation in experimental Trypanosoma rhodesiense infection. (Submitted for 1977 meeting of the American Society of Tropical Medicine and Hygiene).
- 11. Harken, A.H., Lillo, R.S and Haut, M.J. The depressant influence of extracellular fluid hyperoxia on liver slice oxygen uptake. J. Lab. Clin. Med. 89:1269-1277, 1977
- 12. Harken, A.H., Woods, M., Bell, W., Pringle, J.M. and Haut, M.J. Alteration of oxyhemoglobin affinity in canine erythrocytes. (Submitted for 1976 meeting of the Association of Academic Surgeons).
- 13. Johnson, D.J., Djuh, Y-Y., Bruton, J. and Williams, H.L. Improved colorimetric determination of serum zinc. Clinical Chemistry 23:1321-1323, 1977
- 14. Kark, J.A., Haut, M.J., McQuilkin, C.T. and Hicks, C.U. Dissociation of biochemical and hematologic responses to pyridoxine in patients with sideroblastic anemia. Blood 48:966a, 1976 (Presented at American Society of Hematology Meeting, Boston, MA, Dec 1976).
- 15. Kark, J.A., Tarrassoff, P.G., Haut, M.J. and Hicks, C.U. Inhibition of erythrocyte pyridoxal kinase activity (PLK) by a metabolite of isoniazid (INH). Clin. Res. 25: 341A, 1977
- 16. Knodell, R.G., Conrad, M.E. and Ishak, K.G. Development of chronic liver disease after acute non-A, non-B post-transfusion hepatitis: Role of  $\gamma$ -globulin prophylaxis in its prevention. Gastroenterology 72:902-909, 1977
- 17. Knodell, R.J., Kinsey, M.D., Boedeker, E.C. and Collin, D.P. Deoxycholate metabolism in alcoholic cirrhosis. Gastroenterology 71:196-201, 1976
- 18. Knodell, R.G. and Halloway, E.E. Increased biliary excretion of pentobarbital with bile salt-induced hepatic choleresis. Biochemical Pharmacology 25:1682-1684, 1976

- 19. Lorenz, P.E., Tack, B.F. and Schechter, A.N. Radioimmunoassay for the third component of human complement. (7th International Complement Workshop, St. Petersburg, FL, Nov 77).
- 20. Mac Dermott, R.P., Nash, G.S. and Boldt, D. Prearmed effector cells and the target cell specificity of lectin-induced cellular cytotoxicity. J. Immunology 117: 1890-1893, 1976
- 21. Mac Dermott, R.P., Nash, G.S., Saint, J.G., Clark, E.A., Zarai, A.G. and Boldt, D. Autologous human cellular cytotoxicity induced by mitogenic and nonmitogenic lectins. J. Immunology 117:1402-1403, 1976
- 22. Martin, S.K., Okoye, V.C., Hicks, C.U., Esan, G.L., Kark, J.A., Haut, M.J., Williams, A.I., Osunkoya, B.O., Adeniyi, A. and Miller, L.H. Decreased erythrocyte pyridoxal kinase activity (PLK) in blacks: Its possible relation to falciparum malaria. Clin. Res. 25:343A, 1977
- 23. Rickles, F.R., Levin, J., Hardin, J.A., Barr, C.F. and Conrad, M.E. Tissue factor generation by human mononuclear cells: Effects of endotoxin and dissociation of tissue factor generation from mitogenic response. J. Lab. Clin. Med. 89:792-803, 1977
- 24. Salvado, A.J., Speckart, S.F. and Boldt, D.H. Interaction between lectins and CFU-E in plasma clot culture. (Submitted for presentation at ASH meeting Dec 77).
- 25. Scialla, S.J. and Kimball, D.B. Hemostasis studies in patients with colon cancer. (Submitted to VI Int. Cong. Throm. Haem.).
- 26. Scialla, S.J., Speckart, S.F. and Kimball, D.B. The relationship between enhanced platelet aggregation, hypercoagulability and platelet surface sialyltransferase activity in cancer patients. (Submitted for presentation at ASH meeting, Dec 77).
- 27. Steinberg, M.S. and Doctor, B.P. Studies on the effect of 5,5'-dephenylhydantoin on in vitro protein synthesis in rat brain. J. Pharm. and Exp. Therapeut. 198:648-654, 1976
- 28. Thompson, M.R., Steinberg, M.S., Gemski, P., Formal, S.B. and Doctor, B.P. Inhibition of in vitro protein synthesis by shigella dysenteriae 1 toxin. Biochem. Biophys. Res. Comm. 71:783-788, 1976

- 29. Webster, H., Haut, M., Martin, L., Hildebrandt, P. and Gray, I. Blood nucleotide profiles in sequential samples obtained from rhesus monkeys with <a href="Plasmodium">Plasmodium</a> knowlesi. Clin. Res. 25:350A, 1977
- 30. Webster, H.K., Haut, M.J., Hildebrandt, P.K., Martin, L.K. and Gray, I. Changes in blood nucleotide profiles produced by malaria infection by chloroquine prophylaxis, or by chloroquine treatment of infection. (Presented at 1977 meeting, Canadian Federation for Biological Sciences).
- 31. Williams, H., Johnson, D., Haut, M. and Altstatt, L. Structure-activity relationships of aromatic compounds on enzymes involved in heme synthesis. (Submitted for presentation at ASH meeting, Dec 77).
- 32. Williams, H.L., Hooper, D.J. and Haut, M.J. A method for the simultaneous analysis for serum iron and copper using a protein perturbant. Clinical Chemistry 22: 1210a, 1976 (Presented at the 28th national meeting of the American Association of Clinical Chemists, Aug 76).

DESEADOR	AND TECHNOLOGY	WORK HINT S	IMMARY	1. AGER	CY ACCESSIONS	2. DATE OF SU	MMARY	REPORT CONTROL SYMBOL				
					OA 6464	77-10		DD-DR&E(AR)636				
1 0ATE PREV SUMRY 76 10 01	D. Change	S. SUMMARY SCTY*	. WORK SECURITY		ADING <sup>®</sup> DA D	CONTRACTOR ACCESS		ACCESS				
10. NO./CODES:*	PROGRAM ELEMENT	PROJECT	NUMBER	TASK	AREA NUMBER		WORK UNI	THUMBER				
& PRIMARY	61102A	3M161102	BS01		00	141						
b. CONTRIBUTING												
c. CONTRIBUTING	CARDS 114F											
11. TITLE (Precede with Security Classification Code)®												
(U) Pathogenesis of Renal Disease of Military Importance												
SE SCIENTIFIC AND TECHNOLOGICAL AREAS												
012900 Physiology 003500 Clinical Medicine 016200 Stress Physiology 13. SYARY DAYE 118. ESTIMATED COMPLETION DATE 118. FUNDING AGENCY 118. PERFORMANCE METHOD												
54.00		CONT			. 1	1	1	n-House				
54 09 17. CONTRACT/GRANT		CONI		_	DA   C. In-House							
& DATES/EFFECTIVE:					PRECEDING							
& NUMBER:*	IVA			FISCAL	77	9	)	580				
G TYPE:		& AMOUNT:		YEAR	CURRENT							
& KIND OF AWARD:		f. CUM. AMT.			78	9	)	503				
19. RESPONSIBLE DOD	ORGANIZATION			20. PER	FORMING ORGANI	PATION						
MAME: Walter R	eed Army Inst	itute of Re	esearch	HAME:				-C Danamak				
	2			Walter Reed Army Institute of Research								
^00REWashingt	on, D.C. 200	12		Division of Medicine								
				Washington, D.C. 20012								
						PRINCIPAL INVESTIGATOR (Furnish SEAN II U.S. Academic Inelitation)						
RESPONSIBLE INDIVIDUAL					NAME: BUTKUS, COL, D. E. TELEPHONE: 202-576-2265							
NAME: RAPPIUND,	NAME:RAPMUND, COL, G.											
	TELEPHONE: 202-576-3551					SOCIAL SECURITY ACCOUNT NUMBER:						
21. GENERAL USE					ASSOCIATE INVESTIGATORS  NAME: SCHWARTZ, LTC, J. H.							
Foreign intelligence not considered.					NAME: JOHNSON, MAJ, J. P. DA							
Stress; (U) Shock; (U) Fluid and Solute Homeostasis; (U) Dialysis; (U) Kidney Function  23. TECHNICAL OBJECTIVE, 24. APPROACH, 22. PROGRESS (Furnish Individual paragraphs Identified by number procede text of sech with Security Closes/Register Code.)												
23. (U) To	o investigate	mechanisms	for maint	ainir	na fluid.	electro	lyte and	d hemodynamic				
homeostasis	in response	to disease.	injury an	d en	vironment	al stres	ses of	military signi-				
ficance, su	ch as acute re	enal failur	e, shock,	infe	ctious di	sease, h	eat str	ess, and gas-				
trointestin	al disorders	in order to	provide r	ation	nal basis	for pre	evention	and treatment.				
24. (U) C	learance metho	ods, dialys	is, extern	ally	monitore	d isotop	e metho	ds, isotope di-				
lutions, experimental models, in vivo micropuncture, in vitro renal microperfusion, mem-												
brane transport, radioinmunoassay, light and electron microscopy, and chromatography.												
25. (U) 7	610-7709 The	tubuloglom	erular fee	dbacl	k mechani	sm, impo	ortant i	n the patho-				
								n by hemorrha-				
gic hypoten	sion, but not	by aortic	constricti	on,	resulted	in incre	eased tu	buloglomerular				
feedback, suggesting participation of a systemic factor. Examination of the interrela-												
tions of renal vasoactive hormones (prostaglandin, renin and bradykinin) in modulating												
renal nemod	renal hemodynamics and sodium excretion revealed that prostaglandins mediate bradykinin											
induced hyperemia and bradykinin stimulates renin release which in turn "buffers" the												
effects of kinin-induced renal hyperemia. Level of consciousness modifies renal respon- siveness to prostaglandin inhibition and pyrogenic hyperemia. Dithiothreitol was shown												
to block mercuric chloride-induced inhibition of sodium transport in the turtle bladder,												
and to reverse functional changes of mercuric chloride-induced renal failure in dogs												
when given either systemically or by renal artery infusion. Histologic changes in the												
rat proximal convoluted tubule following mercuric chloride correlated with the degree of												
functional impairment and were prevented by dithiothreitol, adding histologic confirma-												
tion to the tubuloglomerular feedback hypothesis. Studies in the turtle bladder demon-												
strated two pathways for phosphate transport, depending upon hydrogen ion concentration.												
For technical report see Walter Reed Army Institute of Research Annual Progress Report												
1 July 1976 - 30 September 1977. 664												
The same of the same of	THE RESERVE OF THE PERSON NAMED IN COLUMN TWO IS NOT THE PERSON NAMED IN COLUMN TWO IS NAMED IN COLUMN TW				E DO FORM	14004 1 1	0 × 44					
DD, 149	AND 1498-1	EDITIONS OF TH	ARMY USE) ARE	OBSOL	ETE.	- 1490A, 1 N	0 7 65					

#### PROJECT 3M161102BS01 RESEARCH ON MILITARY DISEASE

Task Order 00 Internal Medicine

Work Unit 141 Pathogenesis of renal diseases of military importance

Investigators:

Principal: LTC John H. Schwartz, MC

Associates: COL Donald Butkus, MC; John A. Gagnon; MAJ John P.

Johnson, MAJ James Kaufman; Natalie L. Lawson; James S. McNeil; MAJ Lawrence H. Norby; Loutishia Templeman

### Description:

Studies are directed at investigations of mechanisms for maintenance body fluid, electrolyte and hemodynamic homeostasis or their correction in response to disease, injury and environmental stress of heat stress, infectious disease and gastrointestinal disorders. The role of adaptive homeostatic mechanisms, including renal and extra renal mechanisms, whereby body fluid and solute balance is achieved and maintained in the face of stress has been emphasized in order to provide a rational basis for the development of improved methods for prevention and treatment of altered fluid, electrolyte and hemodynamic states and acute and chronic renal failure induced by these stresses.

### Progress:

#### 1. Acute Renal Failure

a. The mechanism responsible for the pathogenesis of acute renal failure, a disease characterized by the sudden loss of renal function, continues to be the subject of controversy. However a majority of recent studies indicate that an alteration in renal hemodynamics, mediated by the renin-angiotensin system are responsible for the near cessation of glomerular filtration and progressive azotemia observed in acute renal failure (1). A schema for the pathogenesis of the hemodynamic change in experimentally induced acute renal failure in animals has been developed from our studies of the effects of heavy metals such as uranyl nitrate (2) or mercuric chloride (3). The initial event is an alteration in transport function of the early segments of the nephron. This epithelial (membrane) transport alteration can be characterized by decreased fluid and electrolyte transport as a direct result of the effect of the nephrotoxin or perhaps ischemia on membrane function (4). As a result of this change in fluid and electrolyte reabsorption, there is an alteration in the composition of the tubular fluid delivered to the macula densa segment of the distal nephron, manifested by an increase in tubular fluid sodium concentration. As a direct consequence of the increased sodium concentration and/or sodium load to the macula densa segment, there is an increase in the renin-angiotensin system activity on an individual nephron level, with an increase in juxtaglomerular renin

activity (4). The increased renin-angiotensin sytem activity mediates a change in renal hemodynamics characterized by a diminution in total renal blood flow with preferential outer cortical ischemia (5). These hemodynamic changes in renal blood flow result in the decline in glomerular filtration (2). Despite the decrease in the filtered load of sodium, as glomerular filtration declines, because of the persistent tubular transport dysfunction of early nephron segments, sodium is delivered to the macula densa segment at high concentrations and the increased renin-angiotensin system activity and altered renal hemodynamics persist (6). Thus, the initiating pathophysiological defect, decreased tubular transport, may also be the cause for the maintenance of the overall decrement in renal functions. The mechanism activated by this increased load of sodium to the macula densa segment as described above can be referred to a tubuloglomerular feedback (4).

 Although the abnormalities in tubular transport function have been observed in various experimental models of acute renal failure, it was not known if they represented the primary alterations as suggested in the above schema or were the result of the almost immediate change in cortical blood flow. Studies were therefore designed to characterize the effects of mercuric chloride and uranyl nitrate on epithelial function. In these studies, in order to avoid the potential change in tubular membrane function which may be associated with perturbations in systemic factors, glomerular filtration, or renal hemodynamics, the effects of these heavy metal salts were studied on an in vitro preparation, the urinary blsdder of the fresh water turtle (Pseudemys scripta) (7, 8). This epithelial preparation, a mesonephric derivative, resembles the mammalian nephron in its capacity to reabsorb sodium, secrete hydrogen ion and respond to aldosterone and vasopressin (9). These studies demonstrated that both uranyl nitrate and mercuric chloride inhibited Na' transport across the short-circuit urinary bladder of the turtle. The inhibition began rapidly after addition of the heavy metal to the urinary side (mucosal) of the membrane, but not after serosal addition. There were no changes in the passive fluxes of electrolytes or tissue electrical resistance. The inhibitory effect could not be reversed by removing the heavy metal from the bulk solution but was rapidly reversed by mucosal addition of dithiothreitol, a compound capable of complexing heavy metals and maintaining monothiols in a reduced state. Lastly, the polyene antibiotic, amphotericin B which increases the apical cell membrane conductance, also reversed the inhibition of Na transport induced by these heavy metals. Our conclusions from these studies were that uranyl nitrate and HgCl, inhibit Na' transport without disrupting the epithelium as a barrier to the passive flow of small ions, and this inhibition is reversible. The most likely site of heavy metal-membrane interaction is within the apical membrane of the epithelial cell and, more than likely, involves complex formation with the sulfhydryl groups of macromolecules associated with Na transport. The net effect of heavy metal is to prevent entry of sodium into the cell from the luminal side without specifically affecting the active, exit step at the basolateral border of the cell or the enzymes required to provide energy for active transport.

- If the schema presented above for the induction of acute renal failure by heavy metals is correct and the effect of the heavy metal salts on tubular transport function is analogous to their effect on the in vitro turtle bladder, dithiothreitol should alter the course of heavy metal induced acute renal failure in a predictable fashion: (1) reduce the natriuresis, (2) prevent the enhancement of juxtaglomerular apparatus renin-angiotensin activity and (3) prevent the decrement in glomerular filtration rate. The effects of dithiothreitol on the course of heavy metal induced acute renal failure were examined in rats exposed to either uranyl nitrate or mercuric chloride (10). As predicted, dithiothreitol prevented the usually observed increased fractional excretion of sodium, azotemia, decreased creatinine clearance and rise in plasma and juxtaglomerular apparatus renin activity after exposure to heavy metal salts. These results support the hypothesis that heavy metals initiate acute renal failure by altering tubular transport function and thereby activating and perpetuating the tubuloglomerular feedback mechanism (10). To further evaluate the effect of dithiothreitol on uranyl nitrate induced acute renal failure a second series of experiments were designed using the dog as the experimental animal. In the earlier studies, using rats, both the heavy metal salts and dithiothreitol were given intramuscularly. It is possible that dithiothreitol altered the induction and progression of acute renal failure by changing the form of heavy metal that the kidney was exposed to rather than a direct effect on the heavy metal - membrane complex within the renal tissue. In the dog studies uranyl nitrate was given as an intravenous dose. After a demonstrable change in renal hemodynamics, dithiothreitol, 2.5 mg/kg body weight, was infused directly into one of the renal arteries. In these studies the changes in renal function on the side not infused with dithiothreitol continued to deteriorate in a characteristic fashion: a decrease in total renal blood flow with a marked cortical ischemia; increased fractional sodium excretion; and a decreased urine volume. The function of the kidney that was infused with dithiothreitol return to control values and by standard clearance techniques appeared to function normally. These studies clearly demonstrate that dithiothreitol not only can prevent but also can reverse the pathophysiological alterations induced by uranyl nitrate. The interaction between the dithiol sugar and the uranium salt must occur within the kidney. These studies exclude the possibility that dithiothreitol acts on some systemic factors since the infusion only improved renal function on the side it was infused.
- d. In addition to these functional studies of heavy metal induced acute renal failure, studies have been designed to assess whether or not cer ain sublethal morphological and histochemical changes known to occur early in the proximal convoluted tubules of mercuric chloride treated rats are related to the onset of acute renal failure. Changes in the proximal tubule of mercuric chloride and mercuric chloride plus dithiothreitol treated rats are being studied by light and electron microscopy and by light microscopic enzyme histochemistry of various marker enzymes. Six time intervals were studied, from one to forty eight hours after mercuric chloride and dithiothreitol were administered. Initially

all segments of the proximal tubule were affected following administration of HgCl, alone. Ultrastructural dispersion of polysomes and increased endocytótic activity was evident in the first and second segments of the proximal tubule ( $P_1$  and  $P_2$  respectively). Terminal  $P_2$  and cortical  $P_3$  ( $P_3$ C) in medullary rays and numerous small apical vacuoles as well as dispersion of polysomes although not as frequent as in  $P_1$  and P, segments of proximal tubule. Small apical dense granules representing lysosomes and multivesicular bodies were seen in Pars Convoluta P2 and P<sub>2</sub>C segment. These cellular changes developed into more extensive lesioñs in later time intervals although at six hours changes in P, tended to appear reversible. At six hours terminal P, and P,C in fiedullary rays showed extensive vacuolar changes that could easily be seen by light microscopy. By electron microscopy numerous vacuoles were seen apically and large cytoplasmic attenuations often projected into the lumen. Dispersion of polysomes and aggregations of smooth endoplasmic reticulum were common features of P2C and P2M. At 12 hours necrosis was evident in pars recta, although, some P<sub>2</sub>M tübules showed varying stages of advanced cell injury. By 24 hours, all cells in P2C and P2M were necrotic as well as the P<sub>2</sub> segment of the pars convoluta. P<sub>1</sub> was less extensively injured and by EM showed varying stages of cell injury compatible with reversible injury. Animals treated with HgCl, + DTT showed the same initial cell changes at one hour as seen with HgCl treated animals, although less extensive. At three hours, dispersed polysomes and apical vacuoles were only observed occasionally. In the remaining time intervals, cellular changes tended to be delayed when compared to the corresponding HgCl, treatment group. i.e. changes seen at six hours in HgCl<sub>2</sub> treatment did not occur until 12 hours in HgCl<sub>2</sub> + DTT treated animals. Nonetheless, at 24 hours, necrosis was evident in pars recta P<sub>3</sub>C and P<sub>3</sub>M, although less extensive when compared to HgCl<sub>2</sub> treated animals. An initial proximal tubular lesion was also demonstrated by enzyme histochemical techniques. Activity for both 5' necleotidase and alkaline phosphatase (marker enzymes for brush border) as well as acid phosphatase (marker for lysomes) was decreased particularly in pars convoluta segments at one hour following HgCl, administration. A transient return to control levels occured at three hours, only to decrease again at six hours when succinic dehydrogenase activity (a marker for mitochondrial inner membrane) is first decreased. A later time intervals, enzyme activity of all enzymes studied tends to be slightly decreased or at control levels in P, reflecting a reversible injury in P, which can be compared to ultrastructural changes. P, and P<sub>3</sub> progressed to slight activity. A protective effect of enzyme<sup>2</sup> activity with the administration of DTT following HgCl, was demonstrated in all time intervals studied. Even during later intervals protection of enzyme activity of terminal P2, P2C, and P2M was evident when compared to HgCl, animals although decreased when compared to controls. These studies demonstrate that there are severe morphological changes in the pars recta segment of the proximal tubule. These necrotic lesions occurred in rats, treated with only mercuric chloride, that had marked decreased renal function and in rats treated with both HgCl, and dithiothreitol that had minimal decrease in renal function. In contrast,

the lesion in the convoluted segments of the proximal tubule appears to correlate with the degree of functional impairment: most marked in  ${\rm HgCl}_2$  rats and minimal in  ${\rm HgCl}_2$ -dithiothreitol treated rats.

- Morphological changes induced in the juxtaglomerular cells by mercuric chloride (HgCl<sub>2</sub>) were also studied using light and electron microscopy. HgCl, was injected subcutaneously into rats at 4 mg per kg body weight and groups of rats were sacrificed at 30 min, 1, 3, 6, and 24 hours after injection. The juxtaglomerular granulation index (JGI) was calculated according to the method of Hartroft and Hartroft (11). Superficial JGI (sJGI), deep JGI (dJGI) and total JGI (tJGI) were determined for each animal. The mean values of all three JGI's were higher in experimental than in control rats. There was an abrupt increase in dJGI and tJGI (P 0.05) at 30 minutes after injection. In experimental rats, the ratios of sJGI/dJGI were lower than control values and gradually decreased over the treatment period with the exception of 30 minutes. The ratios were as follows; 4.3 (control), 2.4 (30 minutes), 3.3 (1 hour), 2.8 (3 hours), 2.0 (6 hours) and 1.2 (24 hours). At 30 minutes after injection, no obvious ultrastructural changes were seen in the juxtaglomerular cells. Ultrastructural changes were observed at 1 hour, suggestive of active formation of granules. By 6 hours granules were present in smooth muscle cells of the afferent arteriole adjacent to the granulated cells. By 24 hours, hypergranulation and hypertrophy of juxtaglomerular granulated cells were observed. The effect of dithiothreitol has not yet been studied. Although the mechanism of the effect of HgCl2-induced acute renal failure is not fully understood, this study further substantiates our hypothesis for the initiation of acute renal failure. We propose that increased renin synthesis and subsequent activation of the renin-angiotensin system, via the mechanism of tubuloglomerular feedback, results in the renal functional disturbances characteristic of acute renal failure and is responsible for the initiation of HgCl\_-induced acute renal failure. The present study supports this hypothesis since increased renin-angiotensin system activity, as judged by JGI, was observed after HgCl<sub>2</sub> administration.
- f. Studies directly assessing the activity of tubuloglomerular feedback in acute renal failure by rat micropuncture are also being pursued. During fiscal year 1977 we completed our preliminary studies of activation of the tubuloglomerular feedback mechanism under conditions of reduced renal perfusion pressure. Our studies of reduced renal perfusion pressure indicate that systemic hypotension results in enhanced feedback responsiveness. That is, there is a more marked decline in single nephron glomerular filtration rate (SNGFR) with increases in tubular fluid flow past the macula densa. However, no enhancement was noted after reductions in renal perfusion pressure caused by aortic clamping. These studies indicate that some factor associated with systemic hypotension activates the feedback response. During the past year we have begun to investigate some of these potential activating factors. In order to do this, we have had to develop a new technique in the micropuncture laboratory. Determinations of SNGFR from proximal and

distal nephron sites give only two points on the feedback response curve, since flow can only be zero or at normal physiologic values. To vary over a wide range and more accurately define the response curve, a means of artificially microperfusing the distal nephron is required. At the same time SNGFR must be estimated at more proximal sites. We have adapted a method of estimating SNGFR from stop flow pressure. This pressure can be recorded continuously using a servonull pressure measuring device with a proximally placed micropipette, while the more distal nephron is microperfused at varying rates with another pipette placed more distally in the proximal tubule than the first pipette. These two sites are separated by an intratubular oil block. We are just beginning to accumulate data using this technique in normal and hypotensive rates.

- g. We have also continued our studies of juxtaglomerular apparatus renin activity. We have demonstrated that volume expansion results in a decline in superficial juxtaglomerular apparatus renin activity. Decreases in renal perfusion pressure result in increases in juxtaglomerular apparatus renin activity, both superficial and deep. However, the increases in deep juxtaglomerula apparatus renin activity was greater after reductions in renal perfusion pressure resulting from systemic hypotension then after aortic clamping. These results would seem to indicate that superficial and deep juxtaglomerular apparatus renin activity may be controlled differentially and respond to a variety of stimuli. Furthermore, increases in renin activity correspond to previously described declines in zonal blood flow in the dog in response to similar stimuli. To study this relationship between changes in juxtaglomerular apparatus renin activity and regional blood flow distribution, we have adapted the use of microspheres to the measurement of renal blood flow in the rat. The method has provided excellent information on total renal blood flow, but has not yet been successfully applied to the measurement of regional blood flow in the rat.
- h. In addition to the apparent central role of the renin-angiotensin system, controlled by the tubuloglomerular feedback mechanism, in acute renal failure other vasoactive-natriuretic agents synthesyzed by the kidney may be important in the pathogenesis of this syndrome. The other major modulator of renal hemodynamics and sodium excretion, endogenous to the kidney is renal prostaglandins. Therefore studies were designed to define the role of prostaglandin on renal function. One method commonly used in other laboratories to define the function of prostaglandins is to infuse these agents and observe their effect on function by clearance techniques. While the infusion of exogenous prostaglandins of the A & E series has been shown to be natriuretic and hyperemic such observations have been subjected to the criticism that an intrarenal infusion does not necessarily mimic its physiological action. To circumvent this problem the administration of prostaglandin synthetase inhibitors have been utilized. Inhibition of the in vivo production of prostaglandins in the anesthetized dog decreases fractional sodium excretion while also reducing renal blood flow. In the conscious

dog, however, renal blood flow is unaltered following prostaglandin inhibition. While this is apparently the most suitable preparation in which to study prostaglandin inhibition, disagreement exists as to whether prostaglandins are natriuretic or antinatriuretic. It has recently been reported that prostaglandin inhibition in the conscious dog produces a natriuresis suggesting that endogenous prostaglandins may therefore be antinatriuretic (12). This data, while compatible with the observation that prostaglandin E (PGE) applied to the serosal side of the toad bladder increased sodium transport, is in general conflict with a larger body of evidence which concludes that prostaglandins are natriuretic. The study which reported an increase in urinary sodium excretion following prostaglandin inhibition was performed in conscious dogs volume expanded with 5% D/W. Since volume expansion is associated with a decrease in proximal sodium reabsorption, such maneuver may be attributed to the natriuresis. Moreover, an increase in the filtered load by glucose leading to glycosuria would be expected to increase sodium excretion due to the increased osmotic effect. The current study was undertaken to determine if the natriuresis so reported may be attributed to the inhibition of prostaglandin synthesis alone or to the combined effects of inhibition plus volume expansion. In one group of conscious dogs volume expanded with 5% D/W (35 ml/kg for 30 min. followed by a maintenance infusion of 1-2 ml/min greater than the urine flow) fractional sodium excretion was examined before and after the i.v. administration of the prostaglandin inhibition, meclofenamate, 2 mg/kg. The same procedure was repeated in a second group of conscious animals not volume exapaded with 5% D/W. The administration of meclofenamate did not significantly alter fractional sodium excretion in either group of animals. It is therefore concluded that prostaglandin inhibition in the volume expanded animal does not induce a natriuresis. Moreover, prostaglandins do not appear to play a major role in the control of sodium excretion in the normal, conscious dog under the described experimental conditions. The role of prostaglandins on renal hemodynamics was also evaluated by examining the effect to pyrogenic agents known to induce renal hyperemia. The intravenous administration of pyrogenic agents such as triple typhoid vaccine results in decreased renal vascular resistance and renal hyperemia. The mechanism responsible for increased renal blood flow following triple typhoid vaccine remains obscure. The similarity of the renal hemodynamic changes following the administration of prostaglandins to those observed during pyrogen induced renal hyperemia suggested the possibility of pyrogenic activation of renal prostaglandin synthesis and subsequent renal vasodilatation. The reported elevations of renal venous prostaglandins or prostaglandin - like material during endotoxin shock lends support to this suggested role for prostaglandins subsequent to triple typhoid vaccine administration. To determine whether pyrogen - induced renal hyperemia is mediated by increased renal synthesis and release of prostaglandins, renal hemodynamics were evaluated in both anesthetized and conscious dogs before and after prostaglandin synthetase inhibition. Our results show that in the anesthetized animal administration of triple typhoid vaccine, during prostaglandin inhibition did not induce the usual renal hyperemic response observed in noninhibited animals.

However, when these experiments were repeated in a group of conscious animals the hyperemic response to the vaccine was not diminished by the prostaglandin inhibition. These results suggest that prostaglandins do not appear to play a major role in modifying renal vascular resistance secondary to triple typhoid vaccine. Failure to observe the typical renal hyperemic response to triple typhoid vaccine in the anesthetized dog following the administration of the inhibitor meclofenamate may be attributed to secondary effects unrelated to prostaglandin synthesis. Since renal blood flow is reduced by meclofenamate in the anesthetized but not conscious dog one might expect this renal vasoconstrictor response to modify or abolish vasodilatory effects of the vaccine, even if the decrease in renal resistance werr related to renal prostaglandins. For this reason prostaglandin inhibitors should not be utilized in studying renal functions in the anesthetized dog. Persistance of renal hyperemia after the administration of meclofenamate in the conscious dog strongly suggests that the renal vasodilatory response to triple typhoid vaccine is not dependent upon the presence of renal prostaglandins.

### 2. Phosphate Homeostasis:

a. As a consequence of stress, acute and chronic renal disease, marked changes in phosphate balance can occur. Since the regulation of bone metabolism, protein synthesis, tissue repair is critically dependent on phosphate levels an understanding of the control processes for phosphate homeostasis is important. The regulation of phosphate levels by renal reabsorption and excretion of this complex anion is a major physiological control mechanism. Although previous studies from this laboratory have characterized the various sites along the nephron involved in phosphate transport (13) little is known about the active cellular processes involved in phosphate transport. We have therefore undertaken studies in a simplified in vitro system to describe some of the factors that are important in the active transport process of phosphate include: (1) a. the pH of the extracellular fluid since phosphate is a week acid and the concentration of the ionic specie transported will vary with pH; and b, the intracellular fate of the inorganic phosphate that enters the cell. Formation of phosphate into organic molecules and formation of high energy phosphate bonds will change the apparent concentration driving forces for the transported ion. The in vitro tissue used in these studies was the urinary bladder of the fresh water turtle. The bladder was mounted in lucite chambers and electrical driving forces were eliminated by continuously short-circuiting the epithelium with an automatic voltage clamp. To eliminate chemical gradients both sides of the epithelium were bathed with an identical turtle Ringers solution. Net Mucosa (M) 3to serosa (S) flux of phosphate (JP) was determined from unidirectional P fluxes across paired hemibladder. The net fluxes were expressed as nanomoles/hr/8cm. The first series of experiments were designed to evaluate the effect of ambient pH on JP. In a solution of the ionic strength of turtle Ringers, the pka of H2PO1 is 6.8. At pH's one-half unit above the pk the predominant ion

species of phosphate present in solution is HPO, at pH's below the pk the predominant specie is HPO<sub>4</sub>. The total amount of phosphate (PO<sub>4</sub>) in the bathing solutions was 5mM. In the absence of exogenous CO<sub>2</sub> and HCO<sub>3</sub> when the Ringers solution was adjusted to pH 6.2 no significant transport could be demonstrated. However, at pH 8.4 JP was 12.1 + 4.8. This suggested that in the absence of  $CO_2/HCO_3$ , the divalent anion,  $HPO_4$ , is selectively transported. Since  $HCO_3$  has been shown to accelerate sodium transport, the effect of HCO, addition on JP was also examined. The isohydric addition of 10mM  $HCO_3$  at pH 8.4 resulted in an increase in JP from 12.1  $\pm$  4.8 to 18.6  $\pm$  4.6. To examine the effect of 10mM  $HCO_3$  at a lower pH, 5% CO, was bubbled through the solution to lower the ambient pH to 7.2. With these imposed conditions, if  $HPO_4$  is the ionic specie transported, JP should decline. However 5%  $CO_2$  addition had no measureable effect on JP. This lack of a decrease in JP suggested that in the presence of CO2 and HCO3 a second transport system may exist for PO4 that transports the univalent anionic specie, HPO,. To evaluate this second transport system for PO, the media conditions were altered such that the pH could be lowered to 6.5 in the presence of both CO, and HCO2. Initially 2.5mM HCO2 was introduced into the solution at pH 8.4 and the measured JP with these imposed conditions was 32.4 + 12. When the pH was lowered to 6.2 by adding 5%  $CO_2$  to the gas phase JP significantly increased to 104.6  $\pm$  32 (P<0.01). If this increment in phosphate transport at this pH was a selective increase in H\_PO\_Atransport and not an effect of PCO, per se, changing the predominant specie of phosphate present in solution to HPO4 by raising the pH, should reduce JP. When the HCO3 concentration was increased from 2.5 to 10mM at constant  $PCO_2$ , 5%, the pH increased to 7.2 and JP decreased by approximately 50%. Thus it appears that there are two transport pathways for phosphate transport. One system utilizes a divalent anion - HPO. This transport system appears to be PCO, independent. A second fransport system utilizes the univalent anion, H2PO4 and is PCO2 dependent.

- b. Effect of PO $_4$  concentration: In order to further describe the transport process or processes, we examined the dependence of Jp on concentration of phosphate at pH 8.4 with 10mM HCO $_3$  and pH 6.5 with 5% CO $_2$  2.5mM HCO $_3$ . Under alkaline conditions, Jp was undectable with 0.56mM PO $_4$ . In 2.5mM PO $_4$  Jp was 12.16 + 4.5 (P< 0.1). This increased significantly to 22.6  $\pm$  8.3 (P< .025) when phosphate concentration was raised to 5mM and further increased to 40.5  $\pm$  9.5 (P< .01) with 10mM PO $_4$ . Thus, at pH 8.4, there was a linear increase in Jp with increase in phosphate concentration. Concentration studies at pH 6.5 have not yet been completed.
- c. Effect of parathormone and acetazolamide: Parathormone causes phosphaturia in many species and decreases Jp in toad bladder. It's action is thought to be mediated by cyclic AMP. We\_studied the effect of PTH and theophylline on Jp at pH 6.5 (2.5 mM HCO $_2$  5% CO $_2$ ) and found no effect. No effect was noted at pH 8.4 (10mM HCO $_3$ ). Similarly Dibutyrl cyclic AMP (5mM) and theophylline (10mM) had no effect on Jp at high or low pH. To test whether the effects of  $CO_2/HCO_3$  addition on Jp

Might be related to their effect on hydrogen ion secretion  $(J_H)$ , studies were carried out with acetazolamide at 10  $^7M$ . In this epithelium acetazolamide at 10  $^7M$  inhibits a large portion of  $J_H$  even in the presence of  $CO_2$ . Under either pH extreme, acetazolamide had no significant effect on Jp (37.1 to 29.7 pH 6.5 and 19.5 to 26.6 at pH 8.4 both not significant).

d. Effect of transport inhibitors on Jp: Since Jp might be related to other active transport processes (either directly as co transport or indirectly as required for turnover of high energy phosphate compounds) we studied the effects of inhibitors under 2 conditions: pH 6.5 (5% CO<sub>2</sub> 2.5mM HCO<sub>3</sub>) and pH 8.4 (10mM HCO<sub>3</sub>). At the acid pH, oubain (10<sub>4</sub>M) significantly inhibited Jp from 28.3 + 6.4 to 7.3 + 4.6 (P <.001) the decrement was entirely due to a decrease in M to S flux. In alkaline conditions, oubain had no significant effect on Jp (42.2 + 22.6 to 35.3 + 23, (P > 0.5). Similar degrees of inhibition of active sodium transport were obtained with DNP, however the decrease in Jp was not as striking. At pH 6.5 Jp decreased from 23.4 + 5 to 13.7 + 3.9 (P $\angle$ .025). Further inhibitor studies are required to differentiate between a primary effect on intermediary metabolism in the cell.

In summary, we have shown that transepithelial transport occurs in this epithelia in the absence of electrochemical gradients. That it is dependent on pH, the  $\rm CO_2/HCO_3$  buffer system and ambient  $\rm PO_4$  concentration and that it is inhibitable by agents affecting other active transport processes. The differing response to pH extremes,  $\rm CO_2/HCO_3$  buffer system and inhibitors suggest that there is more than one mode of transport in this epithelia.

### 3. Acid-Base Homeostasis:

a. The maintenance of extracellular acid base balance is a major function of the kidney. Inappropriate renal responses to stress, sepsis and shock may result in profound systemic acidosis or alkalosis which may be life threatening. In order to devise new modalities of therapy studies have been continued to define the cellular requirements for urinary acidification. These studies have primarily utilized an in vitro preparation, the turtle urinary bladder. Studies have been designed to investigate the energetics of active H transport. The current study examines the relationships between H transport and a specific oxidative pathway of glucose metabolism, the pentose phosphate shunt. To investigate this relationship and metabolic and transport rates were simultaneously measured under several well defined conditions. When H' transport was inhibited by either the application of pH gradients or by acetazolamide, glucose\_metabolism by the pentose shunt declined. Conversly, stimulation of H transport by either imposing a more favorable electrochemical gradient or by CO2 addition resulted in an increase in shunt metabolism. Glycolytic metábolism, in contrast, was invariant with the maneuvers which altered the rate of H' transport. These results suggest that the pentose shunt is an important pathway for H transport. Further studies were also undertaken to define whether or not carbonic anhydrase inhibitors decrease H transport by a direct effect on cellular energy metabolism in addition to their known effect on CO<sub>2</sub> hydration. Since the pentose shunt is required for H transport we examine the effect of acetazolamide and other sulfonamide analogues on glucose-6-phosphate dehydrogenase activity. This enzyme catalyzes the initial step in the shunt pathway and is rate limiting for the pathway. Accetazolamide, 10 M reduced G6PD by 56%. Acetazolamide 10 M increased the km of G6PD for glucose-6-phosphate from 1.0 to 1.8 x 10 M and V max was reduced from 250 to 104 IU/gm protein. At-butyl-analogue of acetazolamide, without carbonic anhydrase inhibitory activity, had no effect on G6PD activity. We conclude that acetazolamide exerts a direct effect on glucose metabolism by inhibiting G6PD. This action may explain the observations that acetazolamide inhibits urinary acidification in dependent of its effect on carbonic anhydrase.

#### PROJECT 3M161102BS01 RESEARCH ON MILITARY DISEASE

Task Order 00 Internal Medicine

Work Unit 141 Pathogenesis of renal diseases of military importance

#### Literature Cited

#### References:

- 1. Flamenbaum, W. Pathophysiology of acute renal failure. Arch. Intern. Med. (Chicago) 131: 911-928, 1973.
- 2. Flamenbaum, W., R. J. Hamburger, M. L. Huddleston, J. S. Kaufman, J. S. McNeil, J. H. Schwartz and R. Nagle. The initiation phase of experiment acute renal failure: An evaluation of uranyl nitrate-induced acute renal failure in the rat. Kidney Internat. 10: S-115-S-122, 1976.
- 3. DiBona, G. F., F. McDonald, W. Flamenbaum, G. J. Dammon and D. E. Oken. Maintenance of renal function in salt loaded rats despite severe tubular necrosis induced by HgCl<sub>2</sub>. Nephron 8: 205-220, 1971.
- 4. Flamenbaum, W., R. J. Hamburger and J. S. Kaufman. Distal tubule (Na<sup>+</sup>) and juxtaglomerular apparatus renin activity in uranyl nitrate induced acute renal failure in the rat: An evaluation of the role of tubuloglomerular feedback. Pflugers Arch. 364: 209-215, 1976.
- 5. Kleinman, J. G., J. S. McNeil and W. Flamenbaum. Uranyl nitrate acute renal failure in the dog: Early changes in renal function and hemodynamics. Clin. Sci. Mol. Med. 48: 9-19, 1975.
- 6. Flamenbaum, W., J. H. Schwartz, R. J. Hamburger and J. S. Kaufman. The pathogenesis of experimental acute renal failure: The role of membrane dysfunction. In: Progress in Molecular and Subcellular Biology, Vol. 5. Editor; F. E. Hahn, Berlin, 1977.
- 7. Schwartz, J. H. and W. Flamenbaum. Heavy metal-induced alterations in ion transport by turtle urinary bladder. Am. J. Phys. 230: 1582-1589, 1976.
- 8. Schwartz, J. H. and W. Flamenbaum. Uranyl nitrate and HgCl<sub>2</sub>-induced alterations in ion transport. Kidney Internat. <u>10</u>: S-123-S-127, 1976.
- 9. Steinmetz, P. R. Cellular mechanisms of urinary acidification. Physiol Rev. <u>54</u>: 890-956, 1975.

- 10. Kleinman, J. G., J. S. McNeil, J. H. Schwartz, R. J. Hamburger and W. Flamenbaum. The effect of dithiothreitol on HgCl<sub>2</sub> and Uranyl Nitrate induced acute renal failure in the rat. Kidney International, In Press. 1977.
- 11. Hartroft, P. M. and W. S. Hartroft. Studies on renal juxtaglomerular cells. I. Variations produced by NaCl and DOCA. J. Exp. Med. 97: 415-429, 1953.
- 12. Kirschenbaum, M. A. and J. H. Stein. The effect of prostaglandin synthesis on urinary sodium excretion in the conscious dog. J. Clin. Invest. 57: 517-521, 1976.
- 13. Hamburger, R. J., N. Lawson and J. H. Schwartz. Fluid absorption and response to parathyroid hormone in defined segments of proximal tubule. Am. J. Physiol. 230: 286-290, 1976.

#### Publications:

- 1. Kaufman, J., R. J. Hamburger, and W. Flamenbaum. Tubulo-glomerular feedback: The effect of dietary NaCl intake. Am. J. Physiol. 231: 1735-1743, 1976.
- 2. Flamenbaum, W., and J. Kaufman. Acute renal failure. The Kidney. November, 1976.
- 3. Flamenbaum, W., J. H. Schwartz, R. J. Hamburger and J. Kaufman. The pathogenesis of experimental acute renal failure: The role of membrane dysfunction. In "Progress in Molecular and Subcellular Biology", Vol. V., ed. F. E. Hahn, Springer-Verlag, Berlin, 1977.
- 4. Kaufman, J., R. J. Hamburger, and W. Flamenbaum. The Effect of volume expansion, hemorrhagic hypotension, and aortic clamping on juxtaglomerular apparatus renin activity. American Society of Nephrology, 1976.
- 5. Flamenbaum, W., R. J. Hamburger and J. S. Kaufman. The effect of alterations in volume status and renal perfusion pressure on superficial and deep juxtaglomerular apparatus renin activity. XII International Congress of Physiological Sciences, 1977.
- 6. Norby, L. H. and J. H. Schwartz. A new mechanism for the inhibition of urinary acidification by acetazolamide. Clin. Res. 24: 443A, 1977.
- 7. Sahib, M. H., J. H. Schwartz and J. S. Handler. Inhibition of sodium transport by carbachol: Potential role of cyclic GMP. Clin. Res. 25: 447A, 1977.
- 8. Johnson, J. P., B. L. Leslie and J. H. Schwartz. Phosphate transport across a urinary membrane in vitro. American Soc. Nephrology, 1976.

9. Norby, L. H., N. Lawson and J. H. Schwartz. Characteristics of pentose shunt dehydrogenase activity in an acid secreting epithelium. American Soc. Nephrology, 1976.

RESEARCH AND TECHNOLOG		Y WORK UNIT	SUMMARY	DAOB 6532 77 10						OL	
76 10 01	D. Change	S. SUMMARY SCTY	U. WORK SECURITY	7. REGR	DING	NL	CONTRACTO	R ACCESS	S. LEVEL OF SU A. WORK UNI		
10. NO./CODES:*	PROGRAM ELEMENT	PROJECT	NUMBER	TASK A	REA HUMBER			IT NUMBER			
& PRIMARY	61102A	3M16110	2BS01		00	00 142					
b. CONTRIBUTING	CARDO 11/E										
c. CONTRIBUTING	CARDS 114F										
(U) Pathophy	ysiology of S		sponses to	Shock	and Tra	auma					
012600 Street	SHNOLOGICAL AREAS®										
13. START DATE	38 Thyslology	14. ESTIMATED COM	15. FUNC	ING AGENCY		16. PERFORMANCE METHOD					
73 07		CONT	DA	i	i	C. In-Ho			-		
17. CONTRACT/GRANT				10. RESC	OURCES ESTIMA	E & PROFES	BIONAL MAN Y	RS & FUR	DE (In thousands	,	
A DATES/EFFECTIVE:	NA	EXPIRATION:			77		1		143		
L NUMBER:				FISCAL	CURRENT		1		143		
C TYPE:		& AMOUNT:		YEAR	78		1		138		
e. KIND OF AWARD:	BCANIZATION	f. CUM. AMT	·	- DE DE	ORMING ORGAN	74700				_	
		L					Tank		<u></u>		
Walter	Reed Army In	stitute of	Research	1	Malter Re	_		ite of	Researc	h	
ADDRESS:*Washing	oton D.C. 2	0012			ivision ∵ Washir			112			
wasiiii)	gcon, b.c. 2	0012			wasiiii	igcon, D	200	112			
				PRINCIPA	AL INVESTIGATE	R (Furnish SEAN	If U.S. Academ	ic Inetitution	,		
RESPONSIBLE INDIVIDU	AL.			PRINCIPAL INVESTIGATOR (Furnish SEAN II U.S. Academic Inetitation)  NAME:  Clagett, MAJ, G.P.							
NAME: Rapmu	ind, COL, G.			TELEPHONE: (202) 576-3793							
TELEPHONE: (202	2) 576-3551			SOCIAL SECURITY ACCOUNT NUMBER:							
21 GENERAL USE				ASSOCIATE INVESTIGATORS							
Foreign inte	elligence not	considere	d	NAME:							
N	SACH with Security Classifi			NAME:							
	5-HIAA; (U)	(0)		tic Activity; (U) Gastric Mucosal Barrier;							
23. TECHNICAL OBJECT	VE. 24 APPROACH, 25.	PROGRESS (Fumish	individual peragraphe ide	identified by number. Precede text of each with Security Classification Code.;							
				activators which may be released from age is aggrevated by the activators which							
										,	
				These studies are important in understanding urs frequently in combat casualties and may							
	rnative metho										
										9	
	etermine if the urinary excretion of 5-HIAA will reflect a simple means of determining lterations in platelet survival. Such studies are preliminary to evaluating the role										
	latelet funci							.6 ,			
	tions known							led in	ito		
	ouches previo										
was then mea	sured. Urina	ary 5-HIAA	excretion v	was m	onitored	in rabb	its who	were	fed		
	lter platele										
and the second second second	0 - 77 09 In								c juice		
	stillations of								_		
Recently, despite use of a variety of commercial fibrinogen preparations, the initial results could not be duplicated. Studies are under way to increase the sensitivity of the assay system before continuing with these experiments. The urinary 5-HIAA ass has been set up and has been found to be reproducible and accurate in measuring 5-HIA in concentrations ranging from 5-50,000 micrograms per milliliter. Preliminary data suggest that this test may be valuable for future platelet survival studies.											
								AA assa	y		
								5-HIAA			
								data			
For technical report see Walter Reed Army Institute of Res						earch An	nual Pr	ogress	Report		
1 July 76 -	30 Sept 77.										
			770								
	re upon originator's eporo		C79								

Work Unit 142 Pathophysiology of systemic responses to shock and trauma

Investigators.

Principal: MAJ G. Patrick Clagett, MC
Associate: COL Norman M. Rich, MC\*
LTC George J. Collins, MC\*\*
John J. Ricotta, MD\*\*\*

# I. <u>Urinary Excretion of 5-Hydroxy Indole Acetic Acid (5HIAA) as an Index of Platelet Survival Time</u>

A. Background and Statement of the Problem. The measurement of platelet survival time has clinical relevance in that it may predict patients predisposed to thrombosis as well as identify those who will benefit from platelet inhibitor therapy. Among the many isotopic methods of labelling platelets for measuring platelet survival, the most widely used is the 51chromium technic. This technic, although highly accurate, is time-consuming, cumbersome, and requires special skills which have retarded its widespread clinical application. Other non-isotopic technics have recently been introduced but have been found, in general, to be unsatisfactory.

It is proposed that the urinary excretion of 5-HIAA might reflect alterations in platelet survival and provide a simple means of its determination. The rationale for this is that platelets release serotonin during aggregation. In vivo, the platelet release of serotonin has been suggested by increased urinary excretion of 5-HIAA, a serotonin metabolite. This has been noted in the postoperative state when platelet survival is shortened. It has also been noted that inhibitors of the platelet release reaction such as aspirin and dextran significantly decrease the postoperative rise in 5-HIAA. These considerations have led us to formulate the hypothesis that changes in platelet survival will be reflected in alterations in urinary excretion of 5-HIAA.

B. Experimental Approach. Rabbits were used to devise a model with shortened platelet survival. Under Innovar anesthesia, a polyethylene catheter was inserted into the acrta via the femoral artery, secured, and left in place. This results in thrombus deposition on the catheter which is non-occlusive and chronically produces a prominent thromboatherosclerotic lesion in the acrtic walls at points of contact with the catheter.

Platelet count, <sup>51</sup>chromium-tagged platelet survival, platelet serotonin, and <sup>14</sup>C-serotonin uptake and release by platelets were determined. For

<sup>\*</sup> Associate Investigator, Chief, Peripheral Vascular Surgery Service, WRAMC

<sup>\*\*</sup> Associate Investigator, Asst. Chief, Peripheral Vascular Surgery Service, WRAMO

<sup>\*\*\*</sup> Associate Investigator, Resident, General Surgery, Johns Hopkins University Hospital, Baltimore, MD

5 days, 24 hour urine collections were obtained and quantitative 5-HIAA excretion assayed.

- C. Results and Discussion. The urinary 5-HIAA assay has been found to be reproducible and accurate in measuring 5-HIAA in concentrations ranging from 1-50,000 micrograms/ml. Baseline measurements in rabbits demonstrate a normal 5-HIAA urinary excretion range of 700-1000  $\mu g/24$  hours. After catheters were placed in rabbits, the mean 24 hours excretion had increased by about 100 mg/24 hours.
- D. <u>Conclusions and Recommendations</u>. Excretion of urinary 5-HIAA is a reliable index of <u>in vivo</u> serotonin release. It is recommended that plans include extension of these studies in other models of shortened platelet survival. One of the other models is the placement of a thoracoabdominal aortic graft made of woven dacron. The design of the graft is such that complete endothelialization does not occur. Thus, the prosthetic surface is continuously exposed to blood. This results in an animal with chronically shortened platelet survival.

#### II. Gastric Juice Fibrinolytic Activity and the Gastric Mucosal Barrier

A. <u>Background and Statement of the Problem</u>. Plasminogen activators are ubiguitous in human tissues and body fluids and injury to such tissues can result in their local release. This may aggravate and promote hemorrhage from an injured tissue, a process known as local fibrinolysis. Local fibrinolysis has been implicated in some cases of upper gastrointestinal hemorrhage. Gastric juice from some patients with erosive hemorrhagic gastritis has been shown to possess fibrinolytic activity consistent with the presence of plasminogen activator, plasminogen, and plasmin.

Hemorrhagic gastritis and stress ulceration are associated with disruption of the gastric mucosal barrier. It is possible that disruption of the gastric mucosal barrier results in the appearance of fibrinolytic activity in gastric juice.

B. Experimental Approach. Dogs with Heidenhain pouches were stimulated to secret gastric juice by the I.V. infusion of histamine HCL 20  $\mu g/kg/hr$ . Gastric juice specimens were collected at timed intervals. A control solution of normal saline and 100 mN HCL is instilled into the pouch and left in contact with the pouch for 1 hour. The solution was emptied and gastric juice samples collected for 1 hour. Then a solution known to disrupt the gastric mucosal barrier was instilled into the pouch and left in place for 1 hour. The pouch was then emptied and gastric juice samples were again collected for one hour.

All gastric juice samples were immediately neutralized by titration with NaOH, placed on ice, and then O.1 ml aliquots are plated onto heated and unheated fibrin plates. The plates were incubated for 18 hours at 37°C and the zones of lysis were measured with a planimeter. The degree of lysis was proportional to the amount of fibrinolytic activity present. Unneutralized

aliquots of gastric juice were analyzed for Na K, Cl, glucose, hemo-globin, and protein concentration.

C. Results and Discussion. Initially it was found that fibrinolytic activity was found in the gastric juice following instillations of sodium taurocholate, sodium salicylate, and ethanol solutions. All of these solutions contained HCL (10-100 mN) and it appeared as one increased the normality of the HCL, more fibrinolytic activity was present in the juice. The fibrinolytic activity was rarely present during control periods (following instillation of acid-saline solution), but consistently appeared following instillation of the barrier breaking solution. The fibrinolytic activity was characterized as a plasminogen activator since it always appeared on unheated plates but not on heated plates (lysis occurring on both heated and unheated plates is indicative of free plasmin or non-specific proteolytic activity).

Following these initially promising results difficulties in the fibrin plate assay developed. This occurred at the time when a new lot of fibrinogen was used to make up the plates. Unfortunately, despite use of a variety of commercial fibrinogen preparations, we have been unable to duplicate our initial results.

D. <u>Conclusions and Recommendations</u>. Our plan is to continue to investigate this problem and refine and increase the sensitivity of the assay system to adequately detect the presence of plasminogen activator before continuing with these experiments.

#### III. Effect of Immuran on Early Patency of Allograft Veins

- A. <u>Statement of the Problem</u>. To determine if Immuran therapy will decrease the rate of allograft thrombosis.
- B. Background. It has been suggested that the difference in patency rate of autografts versus allograft veins may be due to the presence or absence of endothelium, acting as a plasminogen activator, on the luminal surface of the graft. Allograft vessels have been studied in man and a variety of laboratory animals and the histologic changes in the donor vessel following transplantation have been well described. These changes include a marked inflammatory response with endothelial destruction, inflammation and necrosis of the media followed by conversion of the donor vessel to a collagenous tube. The inflammatory process appears to be more marked in arteries than in veins and may be decreased in some instances by administration of immunosupressive agents such as Azathioprine (Immuran). In this setting Azathioprine appears to diminish the inflammatory response of the host to the donor vessel, histologically manifested by a decrease in cellular infiltrate and vessel wall damage. It has been maintained that the ultimate test of a vascular substitute is placement in the venous system. Rich et al. in this institution have established a good model for grafting

in the canine femoral venous circulation. This model should provide the most rigorous test of the effect of azathioprine on allograft patency. Information obtained in this model will be more easily extrapolated to the venous and distal arterial systems of man.

- C. Experimental Approach. Each dog received one autograft and one allograft of external jugular vein (3 cm in length). The jugular vein grafts were prepared identically and randomly returned to the surgeon by an impartial observer. In this way the surgeon was unaware of which vein was an autograft and which was an allograft. A separate record was kept of the source of each graft. In each case allografts were obtained from a male dog. The anastomosis were performed with 6-0 continuous vascular suture after removing 3 cm of femoral vein. Each dog was assigned to one of four treatment groups: 1) No immunosupression, 2) azathioprine 0.75 mg/kg/day, 3) azathioprine 1.5 mg/kg/day, 4) azathioprine 3.0 mg/kg/day. Patency of the grafts was determined by venograms obtained immediately postoperatively and at 1, 2, 3, 5, 7 days and weekly thereafter for 6 weeks or until thrombosis occurs. Both grafts were removed at the time of earliest graft thrombosis and studied by a variety of histologic techniques.
- D. Results and Discussion. Immunosuppression with Immuran in the three dosages described above did not reduce thrombosis of allografts in the femoral venous circulation of host dogs. Since the dosages of Immuran which were used cover the therapeutic range, a further increase in dosage does not appear warranted.
- E. <u>Conclusions and Recommendations</u>. Immuran does not improve allograft patency. Attention should be turned to other drugs for their potential benefit.

#### IV. Effects of Aspirin on Patency of Heterografts in the Venous System

- A. <u>Statement of the Problem</u>. To determine if aspirin will decrease the incidence of early thrombosis and improve the patency of bovine heterografts in the venous system.
- B. <u>Background</u>. Evaluation of patients from the Vietnam Vascular Registry has demonstrated the importance of repairing venous injuries. Recent studies have shown that there is a significant incidence of early thrombosis in venous repairs followed by recanalization. This has implications for acute and chronic venous insufficiency. Several investigators have shown that maintenance of early patency in venous repairs reduces the incidence of limb edema and maintains optimal arterial inflow to the limb. Furthermore, recanalization of thrombosed veins may result in destruction of vein valves and chronic venous insufficiency. Maintenance of early patency thus becomes a matter of great importance in venous reconstructive surgery. Earlier work in this institute has shown that use of bovine heterograft in the venous system is attended by an extremely

high incidence of early graft thrombosis. By using a model known to promote thrombosis, the effects of antithrombotic agents will be more easily demonstrable.

- C. Experimental Approach. Dogs received a bovine heterograft in the femoral vein. Dogs received 600 mg/day of aspirin (treatment group) or no drugs (control group). Vein graft patency was evaluated immediately and at 1, 3, 5, 8 and 14 days postoperatively by venography.
- D. <u>Results and Discussion</u>. In the control dogs, only one graft was patent after two weeks. In the aspirin treated group, six of nine grafts were patent. Aspirin, by virtue of its antiplatelet activity does decrease the incidence of thrombosis and improves patency of heterografts in the venous system.
- E. <u>Conclusion and Recommendations</u>. Aspirin improves patency of heterografts in the venous system over a period of two weeks. Long term studies will be necessary to determine the period of time that an antiplatelet drug needs to be continued to assure long term patency.

Project 3M161102BS01 RESEARCH ON MILITARY DISEASES

Work Unit 142 Pathophysiology of systemic responses to shock and trauma Literature Cited.

### References:

1. Clagett, G.P.: Platelet thromboembolism in human disease. (Submitted)

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY			DAO	A 6467	77 10	01	DD-D	CONTROL SYMBOL R&E(AR)636				
6 10 01	D. Change	S. SUMMARY SCTY	E. WORK SECURITY			NI.	OL SPECIFIC	DATA-	A WORK HOST			
e. NO./CODES:*	PROGRAM ELEMENT	PROJECT	NUMBER	_	REA NUMBER			-				
PRIMARY	61102A	3M161102B	S01		00	143						
. CONTRIBUTING												
. CONTRIBUTING	CARDS 114F											
U) Gastroin  B. SCIENTIFIC AND TE  08800 Life  B. SYARY DAYE  3 09	testinal Resp chhological areas		shock and T			 	G. I	MANCE MET				
7. CONTRACT/GRANT					OURCES ESTIMAT							
A DATES/EFFECTIVE:	NA	EXPIRATION:			PRECEDING							
MUMBER:*				FISCAL	77	2			168			
C TYPE:		4 AMOUNT:		YEAR	CURNENT							
& KIND OF AWARDS		f. CUM. AMT			78							
S. RESPONSIBLE DOD	DREANIZATION			20. PER	FORMING ORGANI	ZATION						
MAME:* Walter Reed Army Institute of Research Division of Surgery ADDRESS:*Washington, D.C. 20012								of Resear				
RESPONSIBLE INDIVIDUAL  NAME: Rapmund, COL, G					PRINCIPAL INVESTIGATOR (Pumlah BEAN II U.S. Academic Institution)  HAME: Reynolds, LTC, D.  TELEPHONE: (202) 576-3791							
TELEPHONE: (202) 576-3551					SOCIAL SECURITY ACCOUNT NUMBER:							
II. GENERAL USE		ASSOCIATE INVESTIGATORS										
					Formeis	ter, MAJ	, J.F.					
oreign inte	lligence not	considered	t	NAME: DA								
		(0)	Gastiic bi									
23 (U) To a control of the control o	constrictors the treatment of the primary constrictors to treatment of the treatment of the primary constrictors are treatment of the primary constrictors were actions were actions were actions were actions were actions were actions to the baboon exceptrine solution of the primary constriction of the constriction of the said for a constillation of the constriction	effects of e. To ste in the dop of gastroin e injected on hepatic s in percendinistered tention were in ere was no liver after regulation anine stome	vagotomy or day the eff g with respected properties of the cartery. Cont cardiac is before an all saline at stilled in change in vagotomy in of gastriach prepara	n gas ect o ect t athol eliac entra outpu d aft 4 de to th gastr n tes c blo	tric bloof intralion the aboogy that artery artery artery to the er vagot grees cene canine ic blood t baboon od flow after ic	od flow, uminal gove para frequer of a bab phere in splanchomy. Do ntigrade stomach flow no s indicatin vagoued salin	, its digastric ameters. atly occupantly occ	stribuccooling These urs in paratis s were . The ration normon hour. ased p shund animal e or to	ution and and se studied a combat ion se also se microns were thermic percentaging play is. The topical			
			(	68 <b>5</b>								

Work Unit 143 Gastrointestinal Responses to Shock and Trauma

Investigators.

Principal: LTC David G. Reynolds, MSC Associate: MAJ Joseph F. Formeister, MC

- I. Regional Distribution and Arteriovenous (AV) Shunting in the Gastric Circulation
- A. <u>Background and Statement of the Problem</u>. Post-traumatic acute gastric ulcerations and attendant upper gastrointestinal hemorrhage pose life threatening complications to combat casualties. Three modalities of therapy are accepted for treatment of this condition.
- 1. Conservative medical management including iced saline lavage with and without the addition of intraluminal vasoconstrictor agents.
- 2. Surgical treatment ranging from vagotomy to total gastrectomy.
- 3. Intra-arterial infusions of vasoconstrictors.

Earlier work in this laboratory noted the effectiveness of epinephrine as a vasoconstrictor on the gastric circulation using the baboon as a test subject. It was concluded that this drug would be a useful alternative to vasopressin for therapy. Other studies failed to demonstrate significant AV shunting in the gastric circulation as the primary mechanism of action of these drugs.

The role of truncal vagotomy in the control of stress ulcer bleeding is postulated to result from the opening of AV shunts in the submucosa which results in directing blood flow away from the mucosa. A study was coordinated to determine the effect of vagotomy on regional blood flow and AV shunting in the gastric circulation of baboons.

With respect to the use of gastric intraluminal cooling and vasoconstrictors, the role of AV shunting is also unclear. Work was therefore undertaken to determine the effect of intraluminal cooling and topical vasoconstrictors on AV shunting with the canine as a model.

B. Experimental Approach. 1. Vagotomy. Total gastric blood flow was measured by an electromagnetic flow transducer placed on the celiac artery of splenectomized baboons who had the common hepatic artery ligated. The hepatic artery was used for the intra-arterial delivery of radioactive microspheres (15  $\pm$  5 u) with three labels. These were injected during a control period,

immediatedy following truncal vagotomy and 2 hours following vagotomy. The localization of the spheres (or failure of the spheres to sequester) in different organ systems was used to determine the regional distribution of blood flow and AV shunting in the baboon stomach. In addition, central injection of microspheres in a different group of test animals was used to determine any change in percent cardiac output to the splanchnic bed after vagotomy. 2. Intragastric agents. Canines were prepared in a manner identical to the previous group of baboon. Central microsphere injections were not performed in these animals. A large diameter synthetic tube was passed into the stomach transorally and a second tube arranged to drain the stomach through a duodenostomy incision. Normal saline at 4°C was delivered through the gastric tube by a roller pump at a fixed rate. In several animals a 250 cc epinephrine and saline solution (6 cc of 1/1000 epineprhine strength solution to 250 cc of normal saline) was instilled into the canine stomach and allowed to equilibrate for one hour. Test dogs received microspheres before iced saline lavage, after start of lavage and 2 hours later. Animals instilled with topical vasopressin solution were given spheres before, after the start of, and 1 hour following the administration of the vasopressin solution.

- C. Results and Discussion. 1. Vagotomy. Results indicate that 80% of the gastric blood flow goes to the mucosa and this is not altered following vagotomy. Less than 5% of the injected microspheres appear in the liver during control periods and this percentage does not appear to change following vagotomy. It would seem that AV shunting has a negligible role in the control of gastric blood flow in a baboon model.

  2. Intragastric agents. Results indicate that approximately 70% of the gastric blood flow goes to the mucosa and this effect is not changed after iced saline stomach lavage or instillation of an epinephrine and saline solution. Less than 1% of injected microspheres appear in the liver during control periods and again the percentage does not change following iced saline lavage or instillation of a topical vasopressor agent.
- D. <u>Conclusions and Recommendations</u>. It would seem that AV shunting is not the mechanism effected by iced saline solutions or topical epinephrine instillation to control gastric hemorrhage in a canine model.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY			DAOA 6527			77 10		DD-DR&E(AR)636				
76 10 01	D. Change	S. SUMMARY SCTY	6. WORK SECURITY			N DISC	B'N INSTR'N L	SPECIFIC D	MO	S. LEVEL OF SUM A. WORK UNIT		
10. NO./CODES:*	PROGRAM ELEMENT	PROJECT	NUMBER	TASK AREA NUMBER				WORK UNIT				
& PRIMARY	61102A	3M1611	102BS01	0	0		144					
b. CONTRIBUTING												
c. CONTRIBUTING	CARDS 114F											
11. TITLE (Procede with	Security Classification Code	,*										
(U) Control	Mechanisms of	f Regional	Circulatio	n								
12. SCIENTIFIC AND TE	CHNOLOGICAL AREAS											
	ical Medicine											
13. START DATE		14. ESTIMATED COM	PLETION DATE	15. FUNDING AGENCY 16. PERFORMANCE METHOD						ноо		
65 07		CONT		DA			1	D. In	-Hous	se		
17. CONTRACT/GRANT	NA			16. RES	OURCES EST		& PROPESSI	ONAL MAN YES	b FUNDS (In thousands)			
& DATES/EFFECTIVE:	NA	EXPIRATION:		PRECEDING			1		122			
L NUMBER:*				FISCAL	77		1		133			
C TYPE:		& AMOUNT:		YEAR	CURRENT							
& KIND OF AWARD:		f. CUM. AMT.			78		1		135			
19. RESPONSIBLE DOD C	RGANIZATION			20. PER	ORMING OR	ANIZA	TION					
MAME: Walter	Reed Army Ins	stitute of	Research	NAME:*	Walter	Re	ed Army	Institu	te of	f Research		
		20012		Division of Surgery								
ADDRESS: Wash	ington, D.C.	20012		ADDRES			on, D.C					
						-						
				PRINCIPAL INVESTIGATOR (Furnish SEAN II U.S. Academic Institution)								
RESPONSIBLE INDIVIDU	AL			MAME: Woods, MAJ, M.								
NAME: Rapt	nund, COL, G.			TELEP			576-235					
TELEPHONE: (20	02) 675-3551			SOCIAL SECURITY ACCOUNT NUMBER:								
21. GENERAL USE				ASSOCIATE INVESTIGATORS HAME:								
Foreign into	lliconco not	considered										
roteign inte	elligence not	Considered										
22. KEYWORDS (Procede	BACH with Security Classific	cation Code)										
	Consumption;		Respirati		10. 0	-	Perfus					
	IVE, 24 APPROACH, 28.											
	efine the mecl											
	elivery and o											
	on oxygen ava											
responses. These studies apply directly to respiratory and hemodynamic support of soldiers with acute battle injuries and/or post surgical stress.									101			
					_							
	lologic techn											
	varieties of	stress. Ti	issue respi	ratio	n, org	an i	metabol	ism and	viab:	ility		
were evaluat												
25 (U) 76 10 - 77 09. Completed studies in an isolated canine heart, hindlimb or												
gracilis muscle indicate that oxygen is relatively unavailable at low temperatures and												
that hypothermic perfusion induces hypoxia. Further, it appears that a thermal												
diffusion probe can be used to monitor perfusion in small volumes of skeletal muscle.												
This will enhance our ability to further evaluate the regional circulations. Spinal												
cord pressor sites have been found over the longitudinal extent of the intermediolateral									diolateral			
nucleus (for pulse, blood pressure and dp/dt). Blood pressure responses are markedly												
	over the uppe											
For technica	al report see	Walter Ree	ed Army Ins	titut	e of R	ese	arch An	nual Pro	gres	s Report.		
1 Jul 76 - 3	The second secon		, 2						J			

Project 3M161102BS01 RESEARCH ON MILITARY DISEASES

Work Unit 144 Control mechanisms of regional circulation

Investigators: MAJ Monty Woods, MC LTC Alden H. Harken, MC

#### I. Factors Influencing Tissue Respiration

A. <u>Background and Statement of the Problem</u>. Oxygen utilization by tissues is influenced by the chemical composition of the perfusing blood and cardiovascular reflexes. Variation in the chemical composition of the blood exerts influences both centrally and peripherally. Experiments were performed in isolated canine hindlimbs which were perfused under tightly controlled conditions. The effects of variation in pH, O<sub>2</sub> and CO<sub>2</sub> content, lactate, and endotoxin on respiration were determined.

- B. Experimental Approach. A membrane lung perfusion system was devised with which a vascularly isolated but viable extremity could be perfused with blood of controlled composition. The chemical composition of the perfusing blood was changed by altering pH, PO<sub>2</sub>, PCO<sub>2</sub>, using stored rather than fresh blood, and adding endotoxin and steroid. The effects of these alterations on tissue respiration were measured.
- C. Results and Discussion. Oxygen consumption ( $V_{02}$ ) was related to the blood's pH by the equation  $V_{02} \approx 100.1$  pH 1643 (r = 0.86); thus a change in acidity of the perfusing blood by 0.1 pH unit causes a 10% change in limb  $V_{02}$ . When blood pH was above 7.3 lactic acid was produced and when pH was below 7.3, it was consumed. The relationship between the A-V lactate difference and blood pH is expressed by Lactate = 22.5 pH 162 (r = 0.75). Lactic acid production does not reflect tissue oxygenation during clinical alkalosis. In seventeen dogs the addition of 5 mg of endotoxin to the perfusate reduced  $V_{02}$  significantly. The eventual addition of 60 mg of methyl-prednisolone returned  $V_{02}$  toward control. Variation of the hemoglobin-oxygen affinity by perfusing the legs with 2-4 week old blood revealed reduced  $V_{02}$  when the  $P_{50}$  of the perfusate was lowered.

These studies demonstrate several relationships between the chemical composition of blood and tissue respiration that are relevant to anesthesia, surgery or blood banking. In addition, evidence is presented in support of steroids being of benefit in shock therapy.

#### II. In Vitro Studies of Liver Respiration

- A. <u>Background and Statement of the Problem</u>. The relationship between extracellular fluid oxygen tension and hepatocyte oxygen uptake was evaluated in 70 rabbits. Normal extracellular fluid (ECF) oxygen tension is 30 torr. It was expected that hepatocyte oxygen uptake would decrease at ECF oxygen tensions below 30 torr presumably due to inadequate diffusion of oxygen into cells. Liver cell oxygen uptake at ECF oxygen tensions above 30 torr was not expected to change.
- B. Experimental Approach. Oxygen consumption of slices of rabbit liver was measured in chambers fitted with oxygen electrodes. The effects of variation of PO<sub>2</sub> in the electrolyte solution and addition of endotoxin on tissue respiration was determined.
- C. Results and Discussion. Oxygen uptake  $(V_{02})$  was maximal at an extracellular fluid PO<sub>2</sub> of 30 torr. At a PO<sub>2</sub> of 10 torr  $V_{02}$  was significantly reduced. However, if PO<sub>2</sub> were increased to 90 torr,  $V_{02}$  was also reduced. These observations indicate that hepatocyte respiration is optimal at low PO<sub>2</sub>s within a narrow range.

The action of endotoxin was evaluated on both slices and homogenates of liver. In both models, endotoxin induced a significant reduction of  $V_{02}$ . It would, therefore, appear that the hepatocyte cell membrane does not protect the cell from the detrimental effects of endotoxin.

# III. Practical Considerations for the Use of a Pulmonary Artery Thermistor Catheter

- A. <u>Statement of the Problem</u>. Much enthusiasm has been generated concerning the clinical value of a pulmonary artery thermistor catheter for the measurement of pulmonary artery wedge (left atrial) pressure and cardiac output.
- B. Experimental Approach. The use of a pulmonary artery thermistor catheter for pressure measurement and thermodilution cardiac output determination was evaluated in eleven dogs. A pulmonary catheter was placed in the wedge position. A left atrial catheter was simultaneously placed via a superior pulmonary vein.
- C. Results and Discussion. Pulmonary artery wedge pressure was a reliable index of left atrial pressure at end expiratory pressures less than 10 cm H<sub>2</sub>O. Fluctuations in pulmonary artery temperature occurred at a frequency equal to the respiratory rate and an amplitude of 0.010°C to 0.086°C. Changes in amplitude were associated with changes in ventilatory waveform, respiratory rate and level of anesthesia. Intermittent and continuous positive pressure ventilation generally dampened and reversed the pulmonary artery temperature pattern exibited during spontaneous breathing. This suggested that when end expiration is used to time indicator injection, cardiac output will be underestimated during spontaneous

breathing and overestimated during continuous or intermittent positive pressure ventilation. When indicator was injected at the same point in the ventilatory cycle, successive values of cardiac output deviated from one another by 0.0 - 6.7%. Deviations as large as 14% resulted if sequential injections were out of phase by half a respiratory cycle. Deviations in measured cardiac output can be minimized by injecting indicator at the same point in the respiratory cycle if it is not feasible to measure cardiac output during apnea. The clinical utility of a pulmonary artery thermistor catheter can be optimized through appreciation of its specific strengths and limitations.

#### IV. Systemic Oxygen Delivery, Oxygen Uptake, and Surgical Stress

- A. Statement of the Problem. Recent advances in respiratory physiology and technology have permitted and promoted endotracheobronchial maneuvering on a scale not previously considered safe. The ventilatory and hemodynamic sequelae of these diagnostic and therapeutic interventions are often not fully appreciated by the operating surgeon. Straight and fiberoptic bronchoscopes at best only partially obstruct large bronchi with resultant decrease in ventilation: perfusion ratio. Conversely, continous postive pressure ventilation may decrease pulmonary blood flow with resultant increase in ventilation: perfusion ratio. The purpose of this study was to evaluate the relationship between systemic oxygen delivery and tissue oxygen uptake during surgical stress.
- B. Experimental Approach. Ten, 25 kg, mongrel dogs were anesthetized with pentobarbital (25/mg/kg). The dogs were intubated and ventilated (15 ml/kg) at a rate of 16 breaths per minute. A femoral artery catheter was placed. Cardiac output was measured by thermal dilution. All parameters (cardiac output, arterial and venous blood gases, pulmonary artery pressure and oxygen consumption) were measured as soon as all catheters were in place (Period #1). Baseline values were again measured 15 minutes following the initial determination (Period #2). All parameters were subsequently measured under the following conditions:
  - Period #3. 10 cm H<sub>2</sub>O positive end expiratory pressure.
  - Period #4. Right thoracotomy with equilibration of intrapleural and atmospheric pressure.
  - Period #5. Right main stem bronchial occlusion with right chest
  - Period #6 Right main stem bronchial occlusion with right chest down.
  - Period #7 Right main stem bronchus released following several deep breaths, right chest up.
  - Period #8 10 cm  $H_2O$  positive end expiratory pressure 100% oxygen (FiO<sub>2</sub> = 1.0).
  - Period #9 Thoracotomy closed, intermittent positive pressure ventilation.

Following the initial thoracotomy, a monitoring catheter (PE 240) was placed into the left atrium of each animal.

C. Results and Discussion. Systemic oxygen delivery (SOD) and oxygen uptake ( $V_{02}$ ) decreased initially in parallel then SOD decreased more than  $V_{02}$ . Following closure of the thoracotomy, SOD was down by 60% and  $V_{02}$  was 80% of control levels. SOD and  $V_{02}$  did not relate to each other in a parallel fashion. Following moderate surgical stress,  $V_{02}$  at the end of the procedure was 80% of control levels.

#### V. In Vitro Studies of Lung Slice Respiration

- A. Background and Statement of the Problem. The relationship between extracellular fluid oxygen tension and lung slice oxygen uptake in the presence of varying concentrations of colloid was evaluated in 86 rabbits. The results described in "II" led to the question of an optimal  $PO_2$  for lung slice oxygen uptake. Also, in vitro data from other labs suggest that the diffusivity of  $O_2$  in solution is strongly influenced by protein concentration.
- B. Experimental Approach. Oxygen consumption of slices of rabbit lung was measured in a Yellow Springs apparatus. The effects of varying  $PO_2$  and the addition of varying concentrations of plasma on lung slice respiration were determined.
- C. Results and Discussion. As with the liver slice experiments, oxygen uptake was maximal at a  $PO_2$  of 30 torr and reduced at  $PO_2$ 's of 10 and 90 torr. There was a direct relationship between the degree of depression of lung slice oxygen uptake and the concentration of protein in the solution. Colloid inhibits oxygen uptake of lung slices. The relevance of these findings to the treatment of shock lung is speculative.

#### VI. Coronary Arterial pH as a Determinant of Myocardial Oxygen Uptake

- A. Background and Statement of the Problem. Acidosis is known to weaken myocardial contraction, decrease heart rate and dilate coronary vessels. Previous work in this laboratory (see I) demonstrated a direct relationship between perfusate pH and skeletal muscle oxygen uptake. The purpose of this study was to examine the magnitude and mechanism of the influence of coronary arterial pH (pHa) on myocardial oxygen uptake (MyO2).
- B. Experimental Approach. A perfused heart preparation was developed which permitted control of heart rate, pressure and flow work, coronary flow, oxygen delivery, pHa and temperature.  $MV_{02}$  and ventricular contractility (dp/dt) could be measured under varying circumstances.
- C. Results and Discussion.  $MV_{02}$  was directly and significantly related to pHa in all animals studied:  $MV_{02}\% = 109$  pHa 743 (r = 0.832).

The mechanism of altered MV $_{02}$  was evaluated by relating dp/dt and MV $_{02}$  to pHa in control, beta-blocked, and potassium arrested hearts. Arterial pH did influence dp/dt and thus MV $_{02}$ : dp/dt% = 199 pHa - 1376 (r = 0.834). This was mediated partially by the effect of catecholamines and pHa: after -blockade dp/dt% = 139 - 929 (r = 0.832). Basal MV $_{02}$  of the perfused potassium arrested heart was also sensitive to pHa: MV $_{02}$  = 1.82 pHa - 12.01 (r = 0.48, p 0.001).

# VII. The Isolated Influence of Temperature on the Ability of Red Blood Cells to Release Oxygen

- A. <u>Background and Statement of the Problem</u>. Temperature influences every aspect of tissue respiration. It has thus been difficult to determine the influence of thermally induced shifts in the oxyhemoglobin dissociation curve because of the strong influence of temperature on tissue oxygen uptake.
- B. Experimental Approach. A membrane lung perfusion system was devised which permitted perfusion of a second membrane lung. This allowed the direct assessment of the influence of temperature on the ability of red blood cells to release oxygen in a metabolically inert system.
- C. Results and Discussion. There is a direct and significant influence of temperature on the ability of red cells to release oxygen. This effect may render cooled tissues hypoxia in the face of adequate blood flow. It is also evident that the evaluation of the metabolic response of tissue to thermal challenge is difficult to assess when their metabolism is hemoglobin dependent.

#### VIII. Alteration of Oxyhemoglobin Affinity of Canine Erythrocytes

- A. <u>Background and Statement of the Problem</u>. There is considerable theoretical evidence that the position of the oxyhemoglobin dissociation curve (ODC) has an influence on oxygen delivery to tissues. Clinical and laboratory documentation has been sparse. As the preferred animal for a large amount of surgical research is the dog, it would be of value to develop a technique for shifting the canine ODC.
- B. Experimental Approach. Dihydroxyacetone, pyruvate, and phosphate were infused into 35 dogs at 1% of blood volume per hour for five hours. One week before or after, a saline solution was infused at the same rate. Hourly 2, 3-DPG, ATP and P50 pre- and post-infusion were measured. Red cell triokinase was assayed.

C. Results and Discussion. Triokinase of canine RBC was 0.2190 1U/gm Hgb (150% normal human level). ATP rose from 1.9 to 2.45 M/gm Hgb after five hrs of infusion of dihydroxyacetone solution. Red cell 2,3-DPG rose from 16.19  $\pm$  0.4 M/gm Hgb to 18.63  $\pm$  0.6 M/gm Hgb after five hours infusion of dihydroxyacetone solution (p < 0.01). P<sub>50</sub> changed from 27.57  $\pm$  0.9 to 31.76  $\pm$  0.6 over the same period (p < 0.001). Saline infusion did not alter 2,3-DPG or P<sub>50</sub>. A simple method of shifting the ODC in awake, unanesthetized dogs has been developed.

# IX. Development of a Thermal Diffusion Probe to Measure Blood Flow in Small Volumes of Tissue

- A. <u>Background and Statement of the Problem</u>. The ability to measure tissue perfusion is currently limited by methods which are discontinuous, unacceptably invasive or both. Attempts at measuring tissue perfusion with the aid of thermal devices has met with limited success. Several methods provide qualitative data but quantification of tissue blood flow has not been realized. An improved technique for measuring thermal conductivity and diffusivity has been developed by engineers working at MIT and Northeastern University. A collaboration with this group has been established with the aim of modifying the technique for direct measurement of tissue blood flow.
- B. Experimental Approach. The thermal diffusion probe was evaluated in an isolated hindlimb preparation which permitted control of flow, temperature and oxygen delivery. Thermal conductivity was measured over a physiologically relevant range of flows.
- C. Results and Discussion. There is a direct and significant relationship between thermal conductivity and tissue blood flow. The initial evaluation of the probe suggests that it can be developed to quantify blood flow in 1 cm<sup>3</sup> volumes of tissue.

#### X. Quantifying Tissue Perfusion From Thermal Properties

A. Background and Statement of the Problem. A previous report from this laboratory (The Physiologist 18:457) suggested that with appropriate modeling, the thermal diffusion probe could be developed to quantify blood flow in small volumes of tissue. A thermal model (probe in tissue) has been developed which discriminates tissue blood flow (w) in gms/cm<sup>3</sup>.sec from measurements of intrinsic thermal conductivity in the absence of flow (k eff) from the expression w =  $(k \text{ eff/km}^{-1})^2 \cdot (km/c_b1^{a2})$  where  $c_b1$  is the heat capacity of blood and a is the probe radius.

- B. Experimental Approach. The purpose of these experiments was to evaluate the relationship of w to flow. The thermal diffusion probe was inserted into the gracilis muscle of an isolated canine hindlimb. The hindlimb preparation permitted control of flow, temperature and oxygen delivery. Limb flow was varied over a physiologically relevant range and w was computed from probe measurements and the expression given above.
- C. Results and Discussion. For 46 measurements in three isolated limbs, w was directly and significantly related to limb flow ( $\dot{Q}$ ): w = 10.9  $\cdot$  10<sup>-6</sup> Q (r = 0.80, p<0.001). Work is in progress to develop an isolated in situ gracilis muscle preparation for further evaluation of the probe.

#### XI. Tissue Hypoxia During Hypothermic Perfusion

- A. <u>Background and Statement of the Problem</u>. Previous work in this laboratory (<u>Fed. Proc. 35:723</u>) isolated the influence of temperature on oxygen availability in a metabolically inert, perfused system and suggested that oxygen may become relatively unavailable to tissues during hypothermic perfusion. The purpose of this study was to evaluate the balance between oxygen supply and demand during hypothermic perfusion of the isolated canine heart.
- B. Experimental Approach. Seventeen dogs were anesthetized with pentobarbital. The heart was isolated and perfused using a membrane lung system which permitted control of temperature, blood gases, pH and coronary flow. Both ventricles were decompressed. Ten hearts were cooled to 16°C and rewarmed to 37°C over two successive periods. Arterial and venous blood was sampled at 4°C intervals for PO2, PCO2, pH, % hemoglobin saturation, and hemoglobin, lactate and pyruvate concentration. Seven control hearts were maintained at 37°C in a time frame matching two successive cooling and rewarming periods. The lactate to pyruvate ratio (LPR) was calculated at 4°C intervals.
- C. Results and Discussion. LPR increased from  $27.9 \pm 3.2$  at  $37^{\circ}$ C to  $35.1 \pm 3.1$  at  $16^{\circ}$ C (p<0.05). During rewarming LPR returned to  $25.3 \pm 2.2$ . There was a significant inverse relationship between LPR and temperature (r = 0.71, p<0.001). LPR of controls did not change during three hours of perfusion at  $37^{\circ}$ C. The results suggest that hypothermic perfusion induces an oxygen supply demand imbalance, rendering tissue effectively hypoxic.

- XII. Distribution of Pressor Sites in the Zona Intermedia of the Spinal Cord of the Cat (Collaboration with MAJ FADEN of Neuropsychiatry)
- A. <u>Background and Statement of the Problem</u>. While there exists a large body of data regarding central nervous system control of cardio-vascular function, the majority of investigations have dealt with afferent connections. Less information exists about efferent pathways and there is very scant data concerning central pathways below the level of the hypothalamus. The purpose of these studies was to determine the precise localization of spinal sympathetic preganglionic pressor neurons.
- B. Experimental Approach. Twenty-six adult cats were studied. The animals were fixed in a Kopf stereotaxis head and spinal unit. Laminectomy was performed to expose thoraco-lumbar segments. A C7 spinal cord transection and bilateral cervical vagotomy were done to eliminate supraspinal and vagal influences. A femoral artery was cannulated for measurement of blood pressure and pulse and the left ventricle was catheterized via the right carotid artery for measurement of peak left ventricular dp/dt. Electrical stimulation of the spinal cord was performed using either a tungsten monopolar electrode or a stainless steel monopolar electrode. Sites of maximal pressor response (SMPR) were identified.
- C. Results and Discussion. Pressor sites were found over all spinal segments between  $T_1$  and  $L_4$ , i.e. the entire longitudinal extent of the intermedio-lateral nucleus. They could not be obtained above  $T_1$  or below  $L_4$ . Over the upper thoracic region, responses were markedly lateralized, the right sided responses being greater than those on the left. Upper thoracic blood pressure changes were exclusively right-sided in some animals. Further work is in progress.
- XIII. Evaluation of an Agent to Decrease the Degree of Sickling at Low Oxygen Tensions (Collaboration with MAJ KARK and LTC HAUT of Hematology)
- A. Background and Statement of the Problem. It has been difficult to find a relatively safe means of pharmacologic protection for patients afflicted with sickle cell disease. The purpose of these studies was the <u>in vitro</u> evaluation of an agent to obtain background information for <u>in vivo</u> testing.
- B. Experimental Approach. Homozygous hemoglobin S blood was used. The red cells were washed and suspended in a phosphate buffered isotonic solution at pH 7.4 at a 10% concentration by volume. The suspension was divided into two fractions, one of which was used for the control. To the other, 30uM of the agent was added. The two fractions were incubated at 37°C for two hours. The fractions were then tonometered at low oxygen tensions and evaluated for morphology and percent of sickled cells.
- C. <u>Results and Discussion</u>. A consistently lower per cent of sickled cells were found in the fraction treated with the agent prior to exposure to low oxygen tensions. Further work is in progress.

Work Unit 144 Control mechanisms of regional circulation

#### Literature Cited.

#### Publications:

- 1. Harken, A.H., Woods, M.: The influence of oxyhemoglobin affinity on tissue oxygen consumption. Ann. Surg. 183:130-135, 1976.
- 2. Bell, W.A., Woods, M., Harken, A.H.: Influence of extracellular fluid hyperoxia and protein concentration on lung slice oxygen uptake. Clin. Res. 24:32A, 1976.
- 3. Woods, M., Harken, A.H., Pringle, J.M., Bell, W.A. (Spon: R.J.T. Joy): The influence of temperature (T) on the ability of blood to release oxygen. Fed. Proc. 35:723, 1976.
- 4. Burleson, R.L., Tilney, N.L., Woods, M.: "The Accumulation of Gallium-67-labelled Leukocytes in Rat Cardiac Allografts." J. Surgical Research, 21:333-334, 1976.
- 5. Bowman, H.F., Woods, M., Balasubramaniam, T.A.: "Thermal Property Measurements in Biological Materials. In <u>Engineering Aspects of Heat Transfer in Medicine</u>, Ed. by J.C. Chato and J.D. Haberman, FSF-University of Illinois Workshop, March 21-24, 1976.
- 6. Harken, A.H., Woods, M., Olsson, R.A.: "Coronary Arterial pH as a Determinant of Myocardial Oxygen Consumption." <u>The Physiologist</u>, 19:218, 1976.
- 7. Woods, M., Harken, A.H.: "Tissue Hypoxia During Hypothermic Perfusion." Fed. Proc. 36:417, 1977.

₩ U. S. GOVERNMENT PRINTING OFFICE: 1978 - 276-330/1433